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e pela Professora Doutora Ana Maria Gonçalves Reis

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Aeromonas spp.: evaluation of genomic diversity and biofilm forming ability

Sara Sofia Pereira Craveiro

This thesis was performed at the Department of Animal Production and Food Safety (DPASA), Faculty of Veterinary Medicine of the University of Lisbon, under the direct supervision of Teresa Maria Leitão Semedo Lemsaddek in Applied Microbiology of the Faculty of Sciences of the University of Lisbon.

This thesis was performed under the supervision of Ana Maria Gonçalves Reis (Faculty of Sciences of the University of Lisbon) and the co-supervision of Maria Teresa Barreto Crespo (Institute of Experimental and Technologic Biology (IBET) and Institute of Biological and Chemical Technology (ITQB).

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“Aquele que nunca viu a tristeza, nunca reconhecerá a alegria.”

(Kahlil Gibran)

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Abstract

Aeromonas spp. are ubiquitous bacteria widely distributed among aquatic environments. Their persistence in water distribution systems is related to their ability to form biofilms, even in the presence of residual chlorine. Once in distribution water systems, aeromonads can contaminate drinking water, food processing facilities and food products. Moreover, members of this genus are known to be responsible for a variety of intra and extra-intestinal diseases in humans, their dissemination occurring essentially through the consumption of contaminated raw food or drinking water.

Since the creation of this genus until the present days, *Aeromonas* taxonomy experienced several changes and numerous approaches have been applied attempting to resolve issues regarding aeromonads species allocation, but this important issue remains under debate.

The present investigation had two main objectives: (i) determine the genomic diversity of aeromonads isolated from food, food processing surfaces, water and clinical samples using a multilocus sequence scheme, previously described and (ii) assess for the biofilm forming ability of a restricted number of isolates representing the microbial collection, and evaluating the effects of three commercial disinfectants on biofilm removal and prevention of biofilm formation.

For multilocus sequence analysis PCR amplification of six housekeeping genes (*gyrB, gltA, groL, metG, ppsA* e *recA)* was performed for 118 *Aeromonas* spp., followed by nucleotide sequencing of the correspondent amplicons. Data analysis demonstrated the high genomic diversity of the isolates under study and further analysis, based on a dendrogram built with the concatenated sequences of five housekeeping genes, allowed aeromonads separation into five well-defined clusters, attributed to the species A*. hydrophila, A. salmonicida, A. caviae* and *A. media*.

Regarding biofilm forming ability, the selected isolates were able to form biofilm on stainless steel coupons, at both refrigeration (4 ^{o}C) and room (20 ^{o}C) temperature. The disinfectants under study demonstrated to be efficient in removing biofilms at both temperatures, but were unsuccessful in preventing biofilm formation.

Overall, the data obtained clearly demonstrated the high genomic diversity of the aeromonads under analysis and also showed promising results regarding species allocation. Furthermore, aeromonads ability to produce biofilm on stainless steel was proved, highlighting the importance of adequate cleaning and disinfection procedures, with emphasis on food processing settings.

Keywords:

Aeromonas spp., multilocus sequence analysis, biofilms, food safety.

Resumo

Os membros do género *Aeromonas* são ubíquos na natureza, podendo ser isolados de uma grande diversidade de ambientes aquáticos e estando associados a uma grande variedade de infecções intestinais e extra-intestinais em humanos e animais.

Desde a sua criação até à atualidade, o género *Aeromonas* tem sido alvo de grande controvérsia, especialmente no que diz respeito à taxonomia. A análise concomitante de características fenotípicas e moleculares é, normalmente, incoerente. A utilização de inúmeras técnicas moleculares tais como hibridação DNA-DNA, sequenciação do rRNA 16S, *Amplified Lenght Polymorphisms* -RFLPs-*, Random Amplified Polymorphic DNA -RAPD-* e *Pulsed Field Gel Electrophoresis* -PFGE-, tem sido levada a cabo com o principal objetivo de ultrapassar esta barreira e, indubitavelmente definir a identificação dos membros deste género a nível de espécie, mas sem grande sucesso.

A presença de aeromonas em ambientes aquáticos está intimamente relacionada com a sua resistência à cloração da água e à sua capacidade para produzir biofilmes. Uma vez sob a forma de biofilme, estes microorganimos tornam-se uma possível fonte de contaminação de águas para consumo e de alimentos, por contacto direto ou por contaminação dos locais de processamento dos mesmos; sendo que a disseminação para o Homem ocorre essencialmente por ingestão de água ou consumo de alimentos crus contaminados.

Assim, o presente estudo teve dois objetivos principais: (i) avaliar a diversidade genómica de *Aeromonas* spp. isoladas de alimentos, ambientes de processamento alimentar, diferentes fontes de água e amostras clínicas, através de *Multilocus Sequence Analysis* e, adicionalmente, avaliar o potencial identificativo desta metodologia; (ii) avaliar a capacidade de formação de biofilme por membros representativos da coleção acima descrita e avaliar a eficácia de três desinfetantes comerciais na remoção de biofilme e na prevenção da formação do mesmo.

De forma a cumprir o primeiro objetivo do trabalho, 118 estirpes de Aeromonas foram submetidas à amplificação de seis genes *housekeeping*, isto é, genes conservados no genoma de *Aeromonas* spp., que codificam para enzimas responsáveis por funções vitais na célula bacteriana. Esta técnica foi recentemente aplicada a membros do género *Aeromonas* por vários autores, embora a escolha dos genes a analisar não tenha sido consensual. No presente trabalho a escolha recaiu sobre o esquema delineado por Martino et al. (2011), que analisa os genes *gyrB, gltA, groL, metG, ppsA* e *recA,* uma vez que foi esta a metodologia que permitiu a criação da "*Aeromonas MLST database*", disponível online através da plataforma NCBI- National Center for Biotechnology Information.

Após amplificação dos genes selecionados por PCR, os respectivos amplicões foram enviados para sequenciação (Macrogen, The Netherlands) e os cromatogramas das sequências recebidas editados de forma a corrigir eventuais erros (SeqTrace, versão 0.81). De seguida, a sequência *forward* foi alinhada com a sequência *reverse* de forma a obter a sequência *consensus*. Posteriormente, uma vez que os *primers* foram desenhados de forma a obter um amplicão com

tamanho superior ao fragmento genómico de interesse, foi necessário proceder à seleção desse fragmento. Para tal, foi realizado o alinhamento de todas as sequências obtidas com algumas das sequências constantes da base de dados *Aeromonas MLST database* de forma a selecionar o fragmento de interesse. De seguida as sequências obtidas foram comparadas com as já existentes na base de dados, de forma a determinar o perfil alélico e o *Sequence Type-ST*. Entre as aeromonas em estudo verificou-se que um elevado número possui novos perfis alélicos e apresenta STs ainda não descritos.

Adicionalmente procedeu-se à elaboração da sequência concatenada utilizando 5 genes, com a exceção de *ppsA* devido a problemas na amplificação/sequenciação. Com base no concatâmero e utilizando o software BioNumerics 6.6 (Applied Maths, Bélgica) foi possível obter um dendrograma em que algumas das estirpes de referência de diferentes espécies -*A. hydrophila, A. salmonicida, A. media* e *A. caviae*- agrupam com isolados da colecção em estudo formando *clusters* bem definidos; o que sugere que as aeromonas agrupadas nesses *clusters* pertençam às espécies acima mencionadas.

Para a concretização do segundo objetivo principal deste estudo, foram selecionados cinco isolados que haviam sido obtidos de origens distintas, nomeadamente locais de processamento de alimentos, água e amostras clínicas, como representantes da coleção anteriormente analisada. Adicionalmente foram introduzidas *A. hydrophila* subsp. *hydrophila* DSMZ 30187 e *Aeromonas aeruginosa* PAO1 como estirpes controlo dos ensaios referentes aos biofilmes.

De forma a avaliar a capacidade de formação de biofilme em discos de aço inoxidável foram estudadas duas temperaturas de incubação, refrigeração (4 ^{o}C) e temperatura ambiente (20 ^{o}C), e períodos de crescimento de 48 h. A quantificação do biofilme formado foi realizada através do cálculo de unidades formadoras de colónias (UFC's).

Os resultados obtidos demonstraram que todas as estirpes em estudo possuem a capacidade de formar biofilme, tanto a 4 como a 20 ^{o}C, não tendo sido detectadas diferenças significativas na formação de biofilme entre estirpes ou temperaturas de incubação.

Subsequentemente foi avaliada a eficácia de desinfectantes comerciais à base de (A) tensioativos anfotéricos, (B) compostos clorados e (C) peróxido de hidrogénio, na remoção de biofilme, bem como a prevenção da sua formação. Os desinfetantes demonstraram ser eficazes na remoção do biofilme, à exceção do desinfetante (A), que foi ineficaz na remoção do biofilme formado pelas aeromonas A31 e S2 (isoladas de um matadouro de suínos e de água de captação do Rio Tejo, respetivamente). Relativamente à inibição da formação de biofilme, avaliada perante a incubação das estirpes na presença dos respetivos desinfetantes, durante 48 h a 20 ^{o}C, nenhum dos desinfetantes demonstrou ser eficaz o que, em contexto real, reflete a possibilidade de concentrações residuais de desinfetante não inibirem a formação de biofilme em superfícies de processamento de alimentos de aço inoxidável, o que constitui um risco agravado para possíveis contaminações cruzadas.

Em conclusão, os resultados obtidos demonstraram a elevada diversidade genómica da população em estudo, o que está de acordo com estudos realizados anteriormente por outros autores.

Adicionalmente, sugere-se que a metodologia utilizada possa ser utilizada na identificação de *Aeromonas* a nível de espécie. No que diz respeito à formação de biofilme e ação de diferentes desinfetantes, os resultados obtidos evidenciam a capacidade de formação de biofilme em discos de aço inoxidável por aeromonas de diferentes origens e da importância deste facto em ambientes de processamento de alimentos, reinterando a importância de adequados procedimentos de limpeza e desinfeção desses locais.

Palavras-chave:

Aeromonas spp., *multi-locus sequence analysis*, biofilmes, segurança alimentar.

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Chapter 1 – General introduction

1.1. Aims of the study

This study takes part of a major project named "AeroPath: Deciphering the pathogenicity potential of food-related *Aeromonas*", carried out by Doutora Teresa Maria Leitão Semedo-Lemsaddek (Universidade de Lisboa, Faculdade de Medicina Veterinária) and Doutora Maria Teresa Barreto-Crespo (IBET – Instituto de Biologia Experimental e Tecnológica).

Briefly, a collection of 118 *Aeromonas* was previously gathered by Barroco (2013), comprising isolates from different sources:

- 70 isolates from Portugal, comprising 20 isolated at a slaughterhouse, 19 isolated from a supermarket, 8 obtained at a cheesemaking factory, 13 isolated from superficial waters from rio Tejo, 4 from food samples and 6 clinical isolates;
- 18 isolates from Belgium, comprising 7 from food samples, 6 from drinking water and 5 clinical isolates;
- 9 clinical isolates from Brazil;
- 6 clinical isolates from Denmark;
- 5 clinical isolates from Bangladesh;
- 3 isolates from Vietnam, comprising 1 from fish, 1 from water and 1 obtained from human stool;
- 1 isolate from Thailand obtained from human stool;
- 6 type strains from *Deutsche Sammlung von. Mikroorganismen und Zellkulturen GmbH* (DSMZ): *A. bestiarum* 13956^T; *A. enteropelogenes* 6394^T; *A. veronii* 7386^T; *A. caviae* 7323^T; *A. hydrophila* subsp. *hydrophila* 30187^T and *A. schubertii* 4882^T.

Aeromonads from the slaughterhouse, the cheesemaking factory and the supermarket were isolated and identified at genus level by Barroco (2013); aeromonads from superficial waters were isolated and identified a genus level by the author of the present study. Isolates identification was undertaken using both phenotypic (Gram, catalase and oxidase tests) and molecular [duplex-PCR described by Marques (2011)] methods. The remaining isolates were gently provided by collaborating laboratories (further details in Appendix A). Isolates from Denmark, Bangladesh, Vietnam, Thailand and Belgium were characterized through several biochemical tests and identified at Hybridization Group (HG) level through, Amplified Fragment Length Polymorphism (AFLP's) and gas–liquid chromatographic analysis of cellular fatty acid methyl esters (FAMEs) (Rahman et al. 2007; Pablos et al. 2011).
In previous work, the diversity of the isolates was assessed by RAPDs with primers OPC19 and 1281 and further evaluated regarding the pathogenicity potential namely antibiotic resistance and virulence profile (Barroco 2013). Additional work regarding adhesion to caco-2 mammalian cells, as well as their invasion ability were also screened by another member of the team.

In the present study we aimed to evaluate the genomic diversity of the collection described above using a multilocus sequence approach previously described by Martino et al. (2011). Additionally, we attempted to determine if this approach had the potential to identify *Aeromonas* at species level.
We also aimed to evaluate the biofilm forming ability of five representative aeromonads isolated from water, food-processing facilities and clinical samples, on stainless steel coupons. Additionally we have assessed the effects of three commercial disinfectants in preformed biofilm removal, as well as in preventing biofilm formation.

The present work is divided into four chapters: Chapter 1 – General introduction; Chapter 2 – Evaluation of genomic diversity among *Aeromonas* spp.; Chapter 3 – Biofilm forming ability of *Aeromonas* spp.; and Chapter 4 – Final considerations.

1.2. General characteristics of the genus *Aeromonas*

Members of the genus *Aeromonas* are essentially ubiquitous in the microbial biosphere and can be isolated from virtually every environmental niche where bacterial ecosystems exist (Janda & Abbott 2010). The genus *Aeromonas* comprises gram-negative straight rods with rounded ends but sometimes they appear as coccobacilli. Cells are 1.0 X 1.0-3.5 µm and, occasionally, can occur singly, in pairs or even as short chains. Most species are motile by means of a single polar flagella; lateral flagella occurs in some species. Even though their optimum growth temperature varies between 22 and 37^{o}C, growth temperature ranges between 0 and 45^{o}C. With regards to pH and presence of NaCl, *Aeromonas* can growth with pH values between 4.5 and 9.0 and 0–4% NaCl. *Aeromonas* spp. are facultatively anaerobic and chemioorganotrophic, displaying oxidative and fermentative metabolism of D-glucose, with gas and / or acid production. They are catalase and oxidase positive, reduce nitrate to nitrite and are enzymatically very active, producing a variety of exoenzymes such as DNase, amylase, chitinase, elastase, esterases, peptidases, arylamidases and other hydrolytic enzymes (Martin-Carnaham & Joseph 2005).

1.2.1 Classification and taxonomy

The genus *Aeromonas* was first described in 1936 by Kluyver and van Niel, being *Aeromonas liquefaciens* originally proposed as the only and type species of this genus. Lately, three more species were recognized as belonging to the genus: *Aeromonas hydrophila* in 1943, *Aeromonas salmonicida* in 1953 and *Aeromonas punctata* in 1957 (Schubert 1967).

In the 7th edition of *Bergey's Manual of Determinative Bacteriology* the genus *Aeromonas* was included in the family Pseudomonadaceae, however, in the 8th edition of the manual it was reclassified into the family Vibrionaceae. Subsequent analyses using 16S rRNA cataloging, 5S rRNA gene sequence comparisons and rRNA-DNA hybridization data revealed that aeromonads demonstrated an evolutionary divergence approximately equidistant from the Enterobacteriaceae and the Vibrionaceae, thereby justifying the reclassification of the genus *Aeromonas* in its own family Aeromonadaceae (Colwell et al. 1986; Martin-Carnaham & Joseph 2005). Further studies based 16S rRNA sequencing have also supported the proposal to recognize Aeromonadaceae as a separate family (Martinez-Murcia et al. 1992).

From the creation of the genus through the 1970's, members of the genus *Aeromonas* could be divided in two groups: the mesophilic motile group, represented by *A. hydrophila,* and the psychrophilic nonmotile group, represented by *A. salmonicida*. In the early 1980's became evident that the mesophilic group was heterogeneous in biochemical and structural properties resulting in the establishment of three phenotypic species, namely *A. hydrophila*, *A. sobria* and *A. punctata/A. caviae*. Attempting to redefine the genus, DNA-DNA hybridization studies have resulted in the recognition of different DNA HG's. According to the 2nd edition of *Bergey´s Manual of Systematic Bacteriology*, the genus includes 14 phenospecies that correspond to 17 DNA HG's. That means that certain HGs cannot be separated at the biochemical level (Carnaham & Joseph 2005; Janda & Abbott 2010).

The number of species attributed to the genus *Aeromonas* has increased during the last decade but in some cases their validity is not universally accepted. Several molecular methods have been applied to highlight aeromonads taxonomical relationships but however, controversies regarding species delineation still remain (Martin-Carnaham & Joseph 2005; Nhung et al. 2007).

Further phylogenetic studies leaded to the reclassification of the existing species and description of novel species. Currently there are twenty seven recognized *Aeromonas* species: A. *punctata/A. caviae*, *A. salmonicida*, *A. hydrophila*, *A. sobria*, *A. media*, *A. eucrenophila*, *A. veronii*, *A. schubertii* , *A. enteropelogenes*, *A. ichthiosmia*, *A. allosachcharophila* , *A. jandaei*, *A. encheleia*, *A. bestiarum* , *A. popoffii, A. molluscorum, A. simiae*, *A. bivalvium, A. aquariorum*, *A. diversa*, *A. fluvialis*, *A. piscicola, A. sanarellii, A. taiwanensis*, *A. tecta*, *A. rivuli*, *A. australiensis* (Euzéby, J.P., 2009. List of prokaryotic names with standing in nomenclature: genus *Aeromonas*. Available at: http://www.bacterio.cict.fr/a/aeromonas.html. Last access on October 2013).

1.2.2. Ecology, etiology and transmission

Aeromonas spp. can be found in all aqueous environments except thermal springs, hyper saline lakes, and extremely polluted waters (Janda & Abbott 1999 in United States Environmental Protection Agency 2006). Aeromonads have been isolated from rivers, lakes, ponds, seawater, groundwater, wastewater, chlorinated drinking water and bottled water and also from various food sources including raw milk, fish, shellfish, raw meats and fresh products (Martin-Carnaham & Joseph 2005; Janda & Abbott 2010). The incidence of *Aeromonas* in water exhibits a seasonal distribution, reaching a higher frequency of isolation in the summer months, due to a major proliferation of the mesophilic strains (Janda & Abbott 2010). Additionally, such distribution is also observed in clinical strains obtained from human stool samples (Sinha et al. 2004).

Members of the genus *Aeromonas* are regarded as the etiologic agents responsible for a wide range of infectious diseases in humans, especially in immunocompromised patients (Janda & Abbott 2010). Gastroenteritis is the disease most frequently associated with aeromonads, which in some cases evolves to cause peritonitis, colitis or colangitis. Additionally, an increasing number of extra-intestinal infections, such as wound, respiratory, genitourinary tract and ophthalmic infections have been documented (Janda & Abbott 1998; Janda & Abbott 2010; Parker & Shaw 2011). Nevertheless, aeromonads are not universally accepted as gastrointestinal pathogens since there are no evidences supporting the conclusion that aeromonads were responsible for a single clonally food-borne disease outbreak. Additionally, the inexistence of a animal model that can trustworthily reproduce *Aeromonas*-associated diarrheal disease inhibits Henle-Koch postulate number three to be fulfilled (Janda & Abbott 2010).

Colonization of the human gastrointestinal tract by aeromonads occurs most likely through the ingestion of contaminated drinking water and food. Additionally, aeromonads can also be acquired by exposure to aquatic environments, thus leading to infection through major or unapparent traumas on skin (Janda & Abbott 2010). Even though less frequently, there have been documented associations

between aeromonads infections and reptile, snake and bear bites (Angel 2002 & Kuminoto 2004 in Janda & Abbott, 2010).

Aeromonas spp. have the potential to resist when chlorine levels are low and form biofilms, even at refrigeration temperatures. The World Health Organization (WHO) listed *Aeromonas* in the third edition of "Guidelines for drinking water quality" [Available at http://www.who.int/water_sanitation_health/dwq/guidelines/en/index.html]. Their persistence in water distribution systems and food-processing environments will consequently act as a source of microbial contamination, increasing the food safety risk (Massa et al. 1999; Béchet & Blondeau 2003; Martin-Carnaham & Joseph 2005; United States Environmental Protection Agency 2006)**.** Therefore justifying the need to assess the potential for biofilm formation of aeromonads isolated from different sources, namely from water, food environments and clinical samples.

Chapter 2 – Evaluation of genomic diversity

2.1. Introduction

Currently, species delineation is based upon the combined information of phenotypic and genomic characteristics of a group of individuals. The concept of species is defined as a group of organisms sharing a set of phenotypic characters, with 70% or higher homology in DNA-DNA hybridization and 16S rRNA gene sequence identity of 97% or more (Mende et al. 2013).

In the genus *Aeromonas*, however, lack of congruence between DNA-DNA hybridization and 16S rRNA gene sequencing have been found (Martinez-Murcia et al. 1992). The high conservation of the 16S rRNA gene sequence (97,8% - 100% of similarity) limits is usefulness for taxonomic analysis at the species level. Moreover, lateral gene transfer and recombination events seem to explain why rRNA operons aren't effectively homogenized thus leading to intragenomic heterogeneity. Restriction Fragment Length Polymorphism (RFLP) of 16S rRNA has been reported as an alternative method but, besides discrepancies between biochemical identification and RFLP patterns, it cannot overcome the intraspecific heterogeneity found among 16S rRNA genes (Borrell et al. 1997; Martin-Carnaham & Joseph 2005; Morandi et al. 2005; Saavedra et al. 2006).

The limited usefulness of 16S rRNA gene sequences suggests that an appropriate identification approach for characterizing *Aeromonas* spp. shouldn't rely on the analysis of a single gene. Thus, a number of other molecular chronometers have been recently used to evaluate phylogenetic relatedness among *Aeromonas* species, including *gyrB* (DNA gyrase, β-subunit), *rpoD* (RNA polymerase), *rpoB* (DNA dependent RNA polymerase, β-subunit) d*naJ* (heat shock protein 40) and *recA* (recombinase A) housekeeping genes (Yanez 2003; Soler et al. 2004; Nhung et al. 2007; Silver et al. 2011). Sequence similarity values of 16S rRNA genes are less divergent (98,7%) than those within the housekeeping genes described above (89% to 92%), which means that their discriminatory power is higher than it is for 16S rRNA (Nhung et al. 2007). Although independent sequence analysis of these housekeeping genes has proved to be an excellent approach for characterizing *Aeromonas* spp., their simultaneous analysis improved the differentiation between closely related species (Soler et al. 2004).

Multilocus sequence analysis

The most widely used molecular typing methods rely on comparisons of DNA fragment patterns on agarose gels, either by (i) DNA restriction - Pulsed Field Gel Electrophoresis (PFGE) and RFLP or (ii) DNA amplification – AFLP and Random Amplified Polymorphic DNA (RAPD), among others.

Due to its high reproducibility and discriminatory power, PFGE is considered the gold standard method; however it is more laborious and expensive than the other techniques. These methods are very useful in short-term epidemiological studies, like for example, establishing clonally relationships among bacteria implicated in a outbreak, however they are not appropriate for long-term epidemiological studies (Cuenca et al. 2013; Pérez-Losada et al. 2013).

Multilocus Enzyme Electrophoresis (MLEE) has a great discriminatory power using genetic variation that accumulates relatively slowly, being appropriate for long-term epidemiological studies. MLEE

analyses the variances in electrophoretic mobilities of housekeeping enzymes and their combination defines the electrophoretic type. Multilocus Sequence Typing (MLST) was proposed in 1998 as an adaptation of MLEE with two outstanding advantages: increased discrimination power and accurate portable data (Maiden et al. 1998).

MLST is based on nucleotide polymorphisms within internal fragments of housekeeping genes. These genes are chosen regarding their high conservation, presence as a single copy and wide distribution across the bacterial chromosome. Fragments of approximately 450 bp to 500 bp are amplified and sequenced, usually for seven housekeeping genes. For each gene, different sequences are assigned as alleles and the alleles of the seven loci are combined into an allelic profile. Each isolate is then unambiguously defined by its allelic profile or Sequence Type (ST) (Maiden et al. 1998; Enright & Spratt 1999). Furthermore, bacterial strains can be clustered based upon concatenated sequences of the set of housekeeping genes. In this case, the term Multilocus Sequence Analysis (MLSA) is more appropriate (Martinez-Murcia et al. 2011).

Although the first MLST scheme was developed as a molecular typing method, its applications have increased. MLST has provide useful data that can be used to evaluate genetic diversity among bacterial pathogens, species delineation, population structure and dynamics, among others(Pérez-Losada et al. 2013).

Over the years, a number of molecular and phenotypic approaches have been applied to characterize aeromonads attempting to achieve a reliable species identification frame. However, despite all efforts, identification of some species is still a serious problem because the conventional biochemical tests in automated or semi-automated commercial systems cannot accurately identify aeromonads to the species level and discrepancies remain between phenotypic and genetic groups (Janda & Abbott 2010).

Two years ago, the first multilocus scheme for studying *Aeromonas* spp. was proposed, attempting to analyze their phylogeny and resolve the taxonomic controversies within the genus (Martinez-Murcia et al. 2011). Meanwhile, two other schemes were developed and, presently, there are three different schemes that altogether comprise the following housekeeping genes: *atpD*, *dnaJ*, *dnaX*, *gyrA*, *rpoD*, *groL*, *metG*, *ppsA*, *recA, gyrB*, *gltA*, *dnaK*, *radA*, *rpoB, tsf* and *zipA* (Martinez-Murcia et al. 2011; Martino et al. 2011; Roger et al. 2012). Furthermore, Martino et al. developed and implemented a Web-based MLST sequence database (http://pubmlst.org/aeromonas) specific for the genus *Aeromonas* based on the analysis of six housekeeping genes: *groL*, *metG*, *ppsA*, *recA, gyrB and gltA*.

In the present study we aimed to evaluate the genomic diversity of a collection of 118 *Aeromonas* isolated from water, food and clinical samples. For this purpose, we applied the previously described multilocus sequence typing scheme developed by Martino et al. (2011). Additionally, we also aimed to assess the potential of this approach for aeromonads species delineation. This scheme of housekeeping genes was chosen mainly due to the existence of a MLST database for comparison purposes. Moreover, the curator of this database (Barbara Cardazzo) is a collaborator of the undergoing investigation.

2.2. Material and methods

2.2.1. Bacterial strains

A collection of 118 aeromonads comprising 70 isolates from Portugal (51 obtained from food and food processing environments, 13 isolated from superficial waters and 6 clinical isolates), 42 isolates from different countries worldwide (8 obtained from food, 7 isolated from drinking water and 27 clinical isolates) and six type strains from DSMZ (*A. bestiarum*; *A. enteropelogenes*; *A. veronii*; *A. caviae*; *A. hydrophila* subsp. *hydrophila* and *A. schubertii)* were analyzed in this study (for further details consult Appendix A). All aeromonads were stored at -80^{o}C in Brain Heart Infusion – BHI – broth (Scharlau, Barcelone, Spain) containing 20% ($^{v}/_{v}$) glycerol and routinely grown on BHI agar at 30^{o}C.

2.2.2. DNA extraction and optimization of PCR amplifications

Genomic DNA for PCR reactions was extracted according to the guanidium thiocyanate method, described by Pitcher et al. (1989). Further quantification of DNA samples in NanoDrop 2000 (Thermo Scientific) allowed concentrations adjustment to 100 ng.

The PCR amplification protocol was optimized according to Martino et al. (2011) using the six type strains described above. Primers used for amplification were developed from the most conserved regions of the genes, in order to obtain amplicons with higher sizes than the sequence of interest. Primers are described in Table 1 and an explanatory scheme of the amplicon and corresponding target sequence sizes is presented in Figure 1.

Table 1- Primers used in PCR amplification reactions

Primers (*)	Sequence	Gene product	Size of PCR amplicon	Size of the target sequence
*gyrB*_F	5' – GGGGTCTACTGCTTCACCAA – 3'			
*gyrB*_R	5' CTTGTCCGGGTTGTACTCGT – 3'	DNA gyrase, β subunit	669 bp	477 bp
*groL*_F	5' – CAAGGAAGTTGCTTCCAAGG – 3'			
*groL*_R	5' – CATCGATGATGGTGGTGTTC – 3'	Chaperonin GroEL	782 bp	510 bp
*gltA*_F	5' – TTCCGTCTGCTCTCCAAGAT – 3'			
*gltA*_R	5' – TTCATGATGATGCCGGAGTA – 3'	Citrate synthase I	626 bp	495 bp
*metG*_F	5' – TGGCAACTGATCCTCGTACA – 3'			
*metG*_R	5' – TCTTGTTGGCCATCTCTTCC – 3'	Methionyl-tRNA synthetase	657 bp	504 bp
*ppsA*_F	5' – AGTCCAACGAGTACGCCAAC – 3'	Phosphoenolpyruvate		
*ppsA*_R	5' – TCGGCCAGATAGAGCCAGGT – 3'	synthase	619 bp	537 bp
*recA*_F	5' – AGAACAAACAGAAGGCACTGG – 3'			
*recA*_R	5' – AACTTGAGCGCGTTACCAC – 3'	Recombinase A	640 bp	561 bp

Legend: (*) according to Martino et al. (2011)

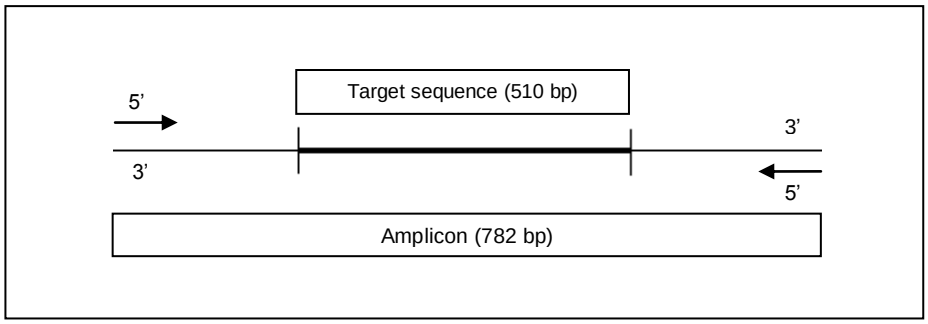

Figure 1 - Representative scheme using *groL* amplicon and target sequence sizes as example.

PCR amplifications were performed in independent reactions using 100 ng of each DNA sample in a final volume of 20 µL of PCR amplification mixture under the conditions described in Table 2.

Table 2 – PCR amplification conditions

Reagents concentrations	PCR amplification conditions (Doppio thermocycler, VWR, Radnor, Pennsylvania, USA)	
1 U of Taq DNA polymerase		94°C, 2 min
(Invitrogen, Life Technologies, Carlsbad, USA)	35 cycles	94°C, 30 s
1X reaction buffer		56°C, 30 s
5 mM $MgCl_2$		72°C, 1 min
0.2 mM of each dNTP		72°C, 5 min
150 mM of each forward and reverse primer		4°C, 10 min

For all the PCR products, 5 µL within 3 µL of a mixture (1:1) of bromophenol blue and GelRed (iNtRON Biotechnology, Korea) were resolved by agarose gel electrophoresis [1% (w/v)] in 0.5X TBE (0.1 M Tris, 0.1 M boric acid, 0.2 mM EDTA) at 110 V for 60 m. On each gel, a molecular weight marker (1 Kb Plus, Invitrogen, Life Technologies) was included at two positions. All gels were visualized and photographed in a UV transilluminator ImageMaster (PharmaciaBiotech, GE Healthcare, United Kingdom). All amplification products were sent for Macrogen Europe (Amsterdam, The Netherands) for sequencing purposes.

2.2.3. Data analysis

Data treatment was performed as follows:

(i) in order to rectify sequencing errors, all chromatograms were edited with SeqTrace software version 0.8.1.;

(ii) for each isolate, a consensus sequence was obtained by the alignment between forward and reverse sequences using the same software;

(iii) sequences of the reference strains used by Martino et al. (2011) were downloaded from the *Aeromonas* MLST database (reference strains are listed in Table 3);

(iv) for each gene, the consensus sequences obtained in "ii" were aligned with the sequences of the reference strains "iii", using Mega 5.1 software;

(v) the target sequences were determined and exported in FASTA format;

(vi) the allelic profile/sequence type of each isolate was assessed using the *Aeromonas* MLST database;

(vii) diversity indices such as G+C content, number of polymorphic sites, Tajima's D test, nucleotide diversity per site (π), average number of nucleotide differences per site (θ) and average number of synonymous and non-synonymous substitutions were calculated using Mega version 5.10;

(viii) for each isolate, the sequences of the housekeeping genes were manually concatenated according to the correct genomic order - *gyrB, groL, gltA, metG, ppsA* and *recA*;

(ix) single sequences and concatenated sequences were introduced in BioNumerics software (version 6.6, Applied Maths NV, Sint-Martens-Latem, Belgium);

(x) construction of dendrograms based on the concatenated sequences of the housekeeping genes and on single gene sequences were performed in BioNumerics. The similarity matrix was obtained by pairwise alignment of the sequences and cluster analysis was obtained by the unweighted pair group method with arithmetic mean algorithm (UPGMA);

(xi) the intra-specific diversity was calculated using Simpson's (D') and Shanon (H') diversity indexes, calculated using the following formulas:

$$D = \sum_{i=1}^{S} \frac{n1\,(n1-1)}{N\,(N-1)} \qquad H = -\sum_{i=1}^{S} \pi_i \log(\pi_i).$$

S – total number of groups formed; N – total number of isolates analyzed; n – number of isolates in the group;

The heterogeneity level is calculated by J', a derivate of Shannon index: $J' = H'/H_{max} = H'/\ln S$.

Table 3 - Reference strains used by Martino et al. (2011)

A. allosaccharophila/A. veronii (CECT 4199^{T})	*A. hydrophila* (CECT 398)
A. bestiarum/A. hydrophila (NCIMB 1134)	*A. media* (DSMZ 4881^{T})
A. bestiarum (DSMZ 13956^{I})	*A. popoffii* (DSMZ 19604^{I})
A. caviae/A. punctata subsp. *caviae* (CECT 838^{I})	*A. salmonicida* subsp. *achromogenes* (NCIMB 1109)
A. caviae (NCIMB 882)	*A. salmonicida* subsp. *masoucida* (NCIMB 2020)
A. encheleia (DSMZ 11577^{I})	*A. salmonicida* subsp. *salmonicida* (NCIMB 1102^{I})
A. enteropelogenes (CECT 4487^{I})	*A. schubertii* (CECT 4240^{I})
A. enteropelogenes/A. trota (CECT 4255^{I})	*A. sobria* (CECT 4245^{I})
A. eucrenophila (DSMZ 17534^{I})	*A. sobria* (NCIMB 75)
A. jandaei (CECT 4228^{I})	*A. veronii* bv. *veronii* (CECT 4257^{I})
A. hydrophila (ATCC 7966^{I})	*A. veronii* bv. *sobria* (CECT 4246)

CECT: Colleción Española de Cultivos Tipo
NCIMB: National Collection of Industrial, Food and Marine Bacteria
ATCC: American Type Culture Collection

In order to assess the reproducibility of the technique, 5% of replicates were performed. For that purpose we used the six type strains described above.

2.3. Results and discussion

In the present investigation we applied a multilocus sequence approach previously described by Martino et al. (2011) to 118 *Aeromonas*.

The isolates were submitted to PCR amplification of six housekeeping genes (Table 1) and the PCR amplification products were further visualized in agarose gels (Figure 2).

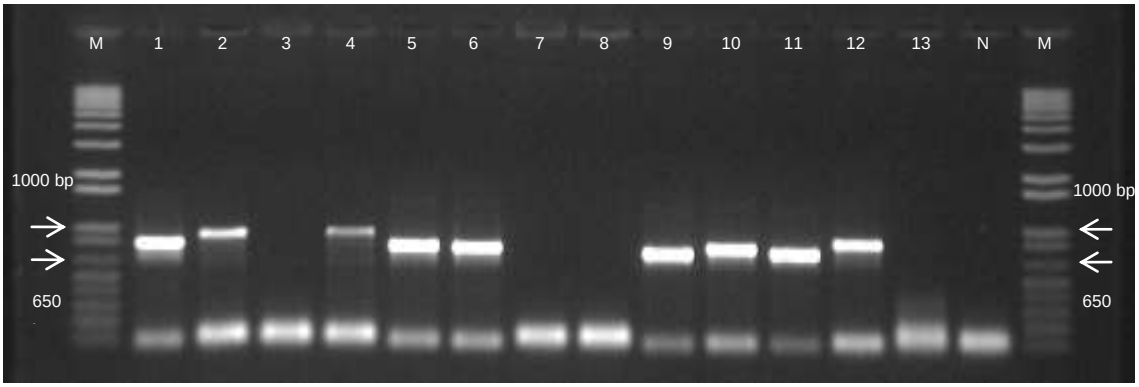

Figure 2 - Amplification products of *recA*. Lanes: 1. A97; 2. A143; 3. A147; 4. A150; 5. A161; 6. A163; 7. A174; 8. A179; 9. A184; 10. A186; 11. A193; 12. A206; 13. A219; N. Negative Control; M. 1 Kb Plus molecular size standard ladder.

Visualization of PCR amplification products allowed the observation of different situations. Although amplicons with expected sizes were observed, most amplicons had higher sizes than expected. For example, amplicons of *recA* should be around 640 bp but, as we can observe in Figure 2, some presented higher sizes such as 850 or 1000 bp. This situation was observed in amplification products of all six housekeeping genes.

Additionally, lack of amplification was also observed in all genes. Attempting to resolve PCR amplification problems several rearrangements were undertaken, including: (i) changes in annealing temperature, ranging values between 56^{o}C and 45^{o}C, and time, from 30 s to 1 min; (ii) changes in $MgCl_2$ concentration (6 mM and 8 mM); (iii) utilization of different enzymes – DYNAZyme II PCR Master Mix (Thermo Fisher Scientific Inc, Langenselbold, Germany), Immolase DNA Polymerase (Bioline, United Kingdom) and NZYTaq 2x Green Master Mix (NZYTech Lda, Lisboa, Portugal), and (iv) use of distinct thermo cyclers – My Cycler^{TM} Thermal Cycler (BioRad, Berkeley, USA) and T3000 Thermo Cycler (Biometra, Goettingen, Germany) and (v) repetition of DNA extractions. Through all these changes, we were able to achieve a larger number of successful amplifications: *gyrB* – 97,76% (113/118); *groL* and *gltA* – 94,07% (111/118); *metG* and *recA* –93,22% (110/118) and *ppsA* – 90,68% (107/118). For further details see Appendix A.

Martino et al. (2011) also reported amplification problems with *ppsA* gene, which did not amplified in *A. salmonicida* subsp. *masoucida*, A. *salmonicida* subsp. *salmonicida* and *A. veronii* bv. *sobria*. Additionally, *A. sharmana* amplified only for *gyrB* gene, which can possibly be explained by the fact that, according to Saavedra et al. (2006), *A. sharmana* does not belong to the genus *Aeromonas.* In a personal communication, Martino et al. reported that they had to change annealing temperature for *gyrB, groL* and *ppsA* genes, which did not amplified in some species, namely *A. jandaei* and *A. enteropelogenes*.

Even though these are highly conserved genes, Martino et al. (2011) reported high nucleotide diversity among them and thus, the major explanation for amplification problems might be the existence of polymorphisms in the primer binding sites.

A total of 624 amplification products, corresponding to 104 isolates *versus* six genes (forward and reverse) were sent for sequencing purposes and 1248 chromatograms were obtained. Through the analysis of the obtained chromatograms, 105 were excluded due to lack of quality. Additionally, through the analysis of the remaining 1143 chromatograms, we confirmed that the sequences obtained had higher sizes than expected, as visualized in agarose gels (Figure 2).

Chromatograms were edited and subsequently forward and reverse sequences were aligned in order to obtain a consensus sequence. Through this alignment, we observed that consensus sequences had approximately the expected amplicon size, depending on the respective gene.

Then, consensus sequences obtained were aligned with *Aeromonas* MLST database sequences of the reference strains representing distinct species in order to obtain the desired target sequence (see Figure 1). Nevertheless, their alignment with the *Aeromonas* MLST database sequences, lead to two different situations: in most cases we were able to obtain the complete allele fragments; however, in other cases the fragments of interest were not complete.

One possible explanation for amplicons unexpected size is that it might be possible that there are insertions of mobile genetic elements between the primer binding sites and the target sequence. Additionally, we did not observe any gaps or insertions within this sequence. Thus, it seems to be more conserved than the upstream region making it a good selection for sequencing purposes.

Thereafter, gene fragments were exported in FASTA format. For those genes which fragments were incomplete, we filled sequences endings with a nucleotide arbitrarily chosen – adenine (A) – so we can avoid a higher loss of sequence data. For example, target sequence of *ppsA* was incomplete in A24 and we filled it as follows: "AAAAAAAAAAAAAAAAACATCATGCGT.....……TTCGGTAGCCCC".

Overall, 24 isolates had complete target sequences, 25 had complete target sequences by the addition of adenine and 31 had complete target sequences for five housekeeping genes (Table 4).

Table 4 - Overview of the number of successful PCR amplifications and sequencing

Source		Total isolates	1)	2)	3)	4)					
						except *ppsA*	except *groL*	except *gyrB*	except *recA*	except *metG*	except *gltA*
Portugal	Slaughterhouse	20	19	2	6	7	0	0	0	0	0
	Supermarket	19	17	6	3	3	0	2	1	0	0
	Cheesemaking factory	8	8	3	3	0	1	1	0	0	0
	Food (INSA)	4	1	0	0	0	0	0	0	0	0
	Superficial waters	13	10	0	4	2	0	1	0	0	0
	Clinical	6	3	3	0	0	0	0	0	0	0
Other countries	Water	7	6	1	0	1	1	0	0	0	0
	Food	8	7	3	1	1	0	0	1	0	0
	Clinical	27	27	6	8	4	3	0	0	1	1
Type strains	DSMZ	6	6	0	0	0	0	0	0	0	0
	Total	118	104	24	25	18	5	4	2	1	1

1) Number of successful PCR amplifications;
2) Number of isolates with complete fragments for the 6 alleles;
3) Number of isolates with complete fragments for the 6 alleles through the addition of "AAA";
4) Number of isolates with complete fragments for 5 alleles.

Sequence types and genetic diversity were assessed by the analysis of the 24 aeromonads with complete target sequences. Allelic profiles were assessed using the *Aeromonas* MLST database and are described in Table 5. In most cases we could not find any match with any allele and, in few cases, some alleles were the same. From the total of 24 aeromonads, 14 had a match with at least one allele representing all new sequence types and 10 didn't have any match, representing sequence types different from those on the database. However, some of these 10 isolates could represent the same sequence type.

For isolates A200 and A258 we were able to allocate 5 alleles. The closest allelic profile encountered in the database was ST 217 for isolate A200, with 3 allelic sites in common (*groL, metG* and *recA*), and ST 189 for isolate A258, with 4 allelic sites in common (*groL, gltA, metG* and *ppsA*). Interestingly, ST 217 corresponds to an isolate from seafood and A200 was isolated from fish. The same correlation was found for ST 189 which corresponds to a clinical strain isolated from the gastrointestinal tract and A258 which is a clinical strain isolated in a Portuguese hospital. These findings suggest that allelic profiles might be identical in aeromonads isolated from the same sample type. However, Martino et al. 2013 observed that the same sequence type not always corresponded to strains isolated from identical samples.

Table 5 - Allele profile and sequence types of 24 aeromonads

Isolates	Species	Source/Origin		Allele					
			ST	*gyrB*	*groL*	*gltA*	*metG*	*ppsA*	*recA*
A 23	*Aeromonas* sp.	Slaughterhouse (surface), Portugal	n	a	a	a	a	122	a
A 28	*Aeromonas* sp.	Slaughterhouse (surface), Portugal	n	a	a	a	a	a	a
A 53	*Aeromonas* sp.	Supermarket (surface), Portugal	n	a	a	a	a	a	a
A 57	*Aeromonas* sp.	Supermarket (surface), Portugal	n	a	116	a	a	a	a
A 61	*Aeromonas* sp.	Supermarket (surface), Portugal	n	102	a	a	a	a	a
A 78	*Aeromonas* sp.	Supermarket (meat), Portugal	n	a	100	a	a	a	97
A 92	*Aeromonas* sp.	Supermarket (surface), Portugal	n	a	3	a	182	a	6
A 93	*Aeromonas* sp.	Supermarket (surface), Portugal	n	156	a	a	165	a	a
A 101	*Aeromonas* sp.	Cheesemaking factory (surface), Portugal	n	1	a	a	a	a	a
A 104	*Aeromonas* sp.	Cheesemaking factory (surface), Portugal	n	a	a	a	a	a	a
A 105	*Aeromonas* sp.	Cheesemaking factory (water), Portugal	n	a	a	a	a	a	a
A 150	*A. caviae* HG4	Clinical, Belgium	n	a	a	159	a	a	a
A 161	*A. salmonicida* HG3	Clinical, Belgium	n	a	6	7	6	a	a
A 172	*A. hydrophila* HG1	Human stool, Belgium	n	a	a	a	a	a	a
A 186	*A. salmonicida* HG3	Drinking water, Belgium	n	a	a	a	a	a	a
A 188	*A. salmonicida* HG3	Food (meat), Belgium	n	123	134	a	a	a	a
A 200	*A. salmonicida* HG3	Fish, Belgium	n	123	191	96	187	a	191
A 202	*A. veronii*	Fish, Belgium	n	a	a	a	a	a	a
A 226	*A. caviae* HG4	Stool, Vietnam	n	a	a	101	198	a	a
A 232	*A. hydrophila*	Clinical, Brazil	n	a	a	a	a	a	a
A 236	*A. veronii*	Clinical, Brazil	n	a	135	a	a	a	a
A 255	*A. hydrophila*	Clinical, Portugal	n	a	a	a	a	a	a
A 258	*A. hydrophila*	Clinical, Portugal	n	a	102	96	96	172	216
A 259	*A. hydrophila*	Clinical, Portugal	n	a	a	a	a	a	a
Allele frequencies			_	4	7	3	5	2	3

n: new ST; a: new allele

In our study, at least 14 out of 24 aeromonads represent all new different sequence types (58%). It also might be possible that the remaining 10 aeromonads represent different sequence types among them. Our results are in agreement with previous publications of different authors. Martino et al. 2011 identified 89 distinct ST's among 96 *Aeromonas* spp. isolated from diseased fish, crustaceans and mollusks (93%), Martino et al. 2013 identified 250 distinct ST's among 258 *Aeromonas* spp. isolated from (97%) and Roger et al. 2012, using another set of housekeeping genes, identified 175 distinct ST's among 191 clinical and environmental strains of *Aeromonas* spp. Additionally, the database contains 252 different sequence types among 272 isolates (93%) [http://pubmlst.org/aeromonas; last access on October 2013]. All these results emphasize the high genetic variability among the genus *Aeromonas*.

To further assess the genetic diversity among the 24 aeromonads, diversity indices were calculated and are represented in Table 6.

Table 6 – Nucleotide diversity observed within the 24 aeromonads

Locus	Fragment size (bp)	GC content (%)	No. (%) of polymorphic sites	dS	dN	Tajima's *D* test	Average no. of nucleotide differences per site (θ)	Sequence conservation
gyrB	477	61%	97 (20,3%)	0,246	0,004	-0,610	0,067	0,797
gltA	495	61,1%	114 (23%)	0,269	0,016	-0,393	0,071	0,770
recA	561	60,0%	126 (22,5%)	0,300	0,004	-0,606	0,073	0,775
groL	510	60,8%	128 (25%)	0,391	0,016	-0,161	0,090	0,749
metG	504	58,8%	135 (26,8%)	0,439	0,029	0,114	0,091	0,732
ppsA	537	64,1%	147 (27,4%)	0,529	0,023	0,355	0,097	0,726
concatenate	3084	61,0%	745 (24,2%)	0,351	0,015	-0,180	0,082	*0,758*

dS: number of synonymous changes per site;
dN: number of non-synonymous changes per site

The mean GC content of the genes varied from 58,8% for *metG* and 64,1% for *ppsA*. The number of polymorphic sites ranged values between 97 polymorphic sites for *gyrB* (20,3%) and 147 polymorphic sites for *ppsA* (27,4%); and the nucleotide diversity (the average number of nucleotide differences per site) ranged values between 0,067 for *gyrB* and 0,097 for *ppsA*. Tajima's *D* test ranged values between -0,610 for *gyrB* and 0,355 for *ppsA*. Tajima's D tests the hypothesis that all mutations are selectively neutral and is based on the differences between the number of polymorphic sites and the average number of nucleotide differences per site (Tajima 1989).

In what concerns the concatenated sequences of the 24 aeromonads, GC content was 61%, the similarity between isolates was 75,8% which corresponds to 745 polymorphic sites and the average number of nucleotide differences was 0,082.

Analysis of the results showed high genetic diversity was found in all genes. Additionally, genetic variance was found to be lower in *gyrB* locus and higher in *ppsA* locus.

Moreover, similar results supporting high genetic diversity were found by (i) Martino et al. 2011 and (ii) Martino et al. 2013: GC content varied from (i) 57,6% for *metG* and 63,7% for *ppsA* and (ii) 58% for *metG* and 64,1% for *ppsA*; the number of polymorphic sites ranged values from (i) 140 for *gyrB* and 233 for *ppsA* and (ii) 162 for *gyrB* and 263 for *ppsA*; nucleotide diversity ranged from (i) 0,057 for *gyrB* to 0,098 for *ppsA* and (ii) 0,058 *gyrB* to 0,106 for *ppsA*; Tajima's D values ranged between (i) -1,109 and -0,336 and (ii) -1,170 and -0.302.

Attempting to clarify the relationships between the isolates of our collection we constructed a dendrogram with the concatenated sequences of 67 isolates five housekeeping genes in their genomic order: *gyrB, groL, gltA*, *metG* and *recA.* Attempting to include a greater number of aeromonads in the dendrogram construction, *ppsA* gene was excluded from the concatenation and

therefore, we were able to construct a dendrogram with 67 concatenated sequences. Additionally, 22 concatenated sequences of the reference strains mentioned on Table 3 were also included.

Considering the dendrogram's global structure and reference aeromonads distribution, a cut off level of 95,8% similarity was chosen. Further analysis allowed the visualization of five well defined clusters attributed to *A. salmonicida* (A)*, A. hydrophila* (B)*, A. caviae* (C and D) and *A. media* (E) (Figure 4).

A. salmonicida cluster included *A. salmonicida* subsp. *salmonicida* (NCIMB 1102^T), *A. salmonicida* subsp. *masoucida* (NCIMB 2020), *A. salmonicida* subsp. *achromogenes* (NCIMB 1109), isolates A57, A61, A62, A184, A186, A188, A193, A195 and A200.

A. hydrophila cluster included ATCC 7966^T, A85, A97, A98, A101, A105 and A163 A172, A232, A237 and A255. Accordingly, this last group of isolates was previously identified as *A. hydrophila* HG1.

Two different clusters were formed for the species *A. caviae*. Cluster C included type strain CECT 838^T, A78, A104, A258, S1, S3, S5, S6, A147, A150, A154, A157, A219, A222, A226, A230 and A253. Accordingly, isolates A147 to A230 were previously identified as *A. caviae* HG4. Clinical isolate A258 were previously identified as *A. hydrophila*, however, in clinical laboratories, aeromonads identification is based on their biochemical properties (Janda & Abbott 2010) rather than molecular methods. Once molecular methods are more reliable in aeromonads species allocation than biochemical methods, it is most likely that A258 is *A. caviae* rather than *A. hydrophila*. The other cluster (D) included NCIMB 882, A4, A5, A7, A8, A23, A24, A25, A27, A28, A31, A52 and A53. Interestingly, all these were isolated from the slaughterhouse, with the exception of A52 and A53 that were isolated from the supermarket. This is the only situation where isolates were clustered according to their source.

Finally, *A. media* cluster included type strain DSM 4881^T and isolates A92, A95, A99, A161 and S10. A161 were previously assigned as *A. salmonicida* HG3, however, it clustered with *A. media* DSM 4881^T with 99,6% similarity. This situation needs to be further evaluated using, for example, DNA-DNA hybridization studies.

In the absence of representative isolates of other species, some reference strains were found in the same clusters.

A major cluster constituted by *A. veronii* bv. *sobria* CECT4246^T, *A. veronii* bv. *veronii* CECT4257^T, *A. sobria* NCIMB75, *A. hydrophila* and *A. allosacharophila*/*veronii* CECT4199^T was observed. In this cluster, isolate A259, previously identified in a clinical laboratory as *A. hydrophila*, clustered with *A. sobria* NCIMB75 with 96% similarity. This situation can be explained exactly as the situation found for isolate A258 and reinforces the idea that biochemical characterization is not suitable for aeromonads species allocation. Additionally, isolate A94 clustered with *A. allosacharophila*/*veronii* CECT4199^T with 99,4% similarity. Isolate A202, previously identified as *A. veronii*, clustered with *A. sobria* CECT4245^T. This result should be further confirmed.

Isolate S13 clustered with *A. eucrenophila* DSMZ17534^T with 96,8% similarity, which means that this isolate should probably be allocated to *A. eucrenophila* species.

Isolate A231, previously identified as *A. trota* clustered with *A. enteropelogenes* CECT4487^T and *A. enteropelogenes/trota* CECT4255^T with 96,2% similarity. Since A. trota is an earlier heterotypic synonym of *A. enteropelogenes*, previous identification was confirmed by our study.

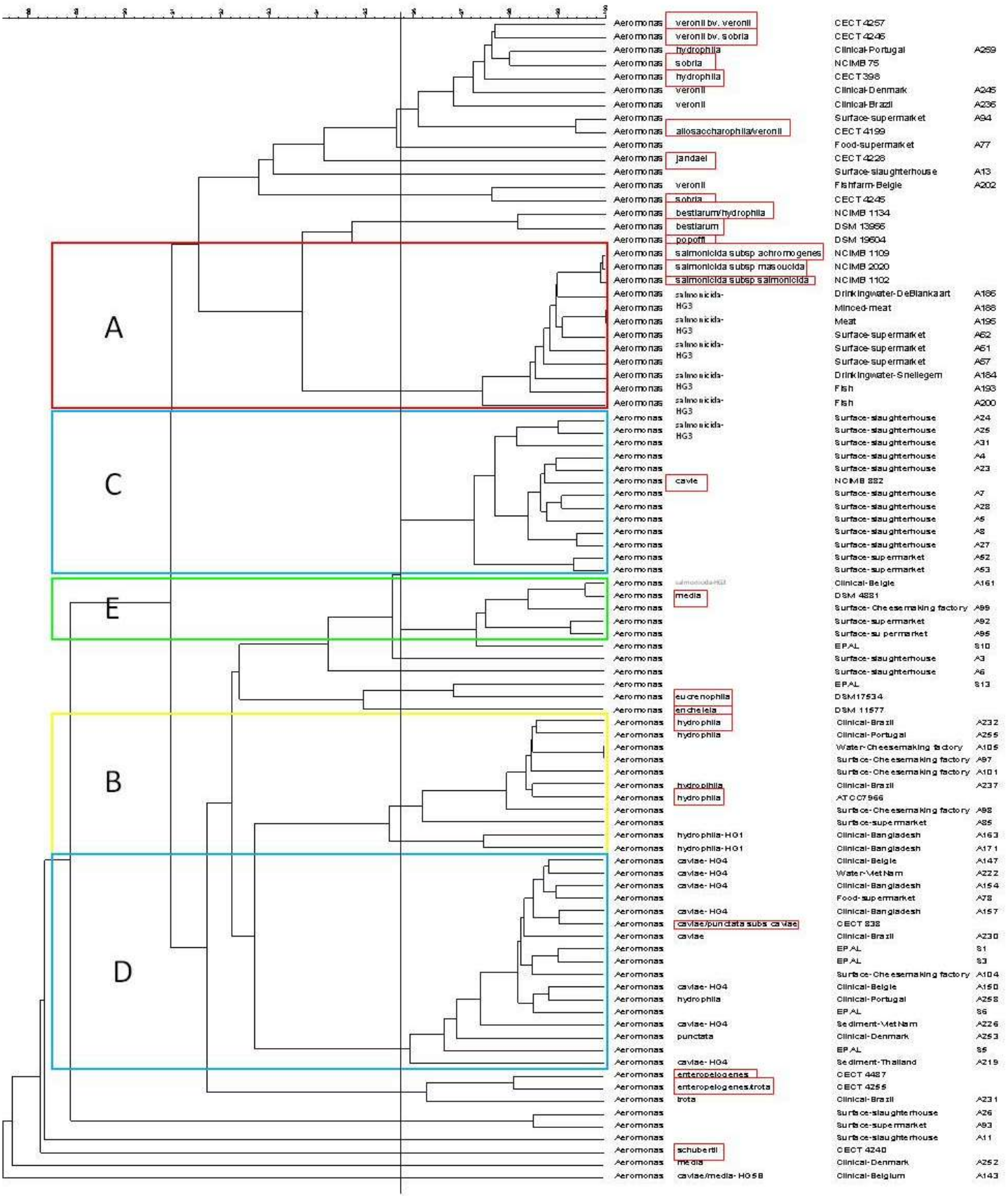

Figure 3 - Dendrogram obtained by multiple alignment of the concatenated sequences of five housekeeping genes grouped by the agglomerative clustering of unweighted pair group method with arithmetic mean (UPGMA).

Red rectangle (A)*: A. salmonicida* cluster; Blue rectangle (C,D): *A. caviae* clusters; Green rectangle (E): *A. media* cluster; Yellow rectangle (B): *A. hydrophila* cluster.

A. bestiarum/hydrophila NCIMB1134 formed a cluster with *A. bestiarum* DSMZ13956^T; single clusters were observed by *A. jandaei* CECT4228^T, *A. popoffi* DSMZ19694^T, *A. encheleia* DSMZ11577^T, *A. schubertii* CECT4240^T, A3, A6, A11, A13, A77, A143 and A252. Finally, A26 and A93, isolated from a supermarket and a slaughterhouse, clustered with 98,4% similarity, which means that they should probably be allocated to the same species; however, none of the represented in the present study.

The inclusion of isolates representative of these species should allow the respective formation of well defined clusters. Additionally, it should also be included reference strains representing other *Aeromonas* species.

Additionally, to create a comparison between the six housekeeping genes and concatenated sequences, dendrograms based on single gene sequences were constructed (Appendix B). These dendrograms were constructed with sequences of the 22 reference strains (Table 3) and the 49 aeromonads with complete gene fragments. All six dendrograms formed the same five clusters as did the dendrogram of the concatenated sequences, even in the absence of *ppsA* gene. These findings suggest that all six genes would be able to distinguish members of these four species, even if they weren't used combined. It also supports the species identification based on the five well defined clusters.

Single dendrograms based on the sequences of six type strains of the aeromonads collection (Appendix A) and the sequences of the 22 reference strains downloaded from the database (Table 3) were constructed. All six type strains used in the present study are from DSMZ; however, they all had homology with six of the 22 reference strains used by Martino et al. (2011). Dendrograms construction (data not shown) allowed the observation of clusters formed by homologous type strains sequences, with 100% similarity, thus conferring credibility to the technique.

Simpson's diversity index (D) is the probabilities of two randomly selected individuals belonging to the same group. Nonetheless, D is inversely proportional to diversity and the complementary (D') needs to be calculated to assess the probability of two randomly selected individuals belong to different groups. Samples diversity are considered acceptable if D>0,90 and the maximum possible value is "1", which corresponds to one individual per group (Hunter & Gaston 1988). Simpson's diversity obtained was D' = 0,906, which means that the sampling analyzed allow the isolation of distinct individuals.

Shannon's diversity index (J') measures population's heterogeneity. It takes into account both abundance and evenness of species present in the community. Shannon's diversity obtained was J'=0,83, which highlight population diversity.

2.4. Conclusions

In the present study we aimed to (i) evaluate the genomic diversity among a collection of 118 *Aeromonas* using a previously described multilocus sequence typing scheme and (ii) assess the potential of this approach in aeromonads species allocation.

Genetic diversity was found to be high in the population analyzed. In a total of 24 aeromonads, none had known STs and at least 10 new different ST's are proposed. Additionally, several diversity indices confirmed the high level of sequence diversity detected.

The MLST scheme used allowed the separation of five well defined clusters attributed to *A. salmonicida, A. hydrophila, A. caviae* and *A.media.* This revealed to be a valuable tool in what regards to *Aeromonas* species allocation, however, the inclusion of representative isolates of other species would provide more reliable results.

Chapter 3 – Biofilm forming ability

3.1. Introduction

Aeromonas spp. have been isolated from chlorinated drinking water supplies in several countries (United States Environmental Protection Agency 2006) even during colder months, with temperatures below 14°C, and in food held at refrigerated temperatures (Massa et al. 1999; Manuel Pablos et al. 2009). Even though conventional water treatment processes are effective in removing or inactivating aeromonads, they may persist in distribution systems as biofilms when disinfectant levels are low (< 0.2 mg/L free chlorine residual). Aeromonads ability to survive and multiply at low temperatures and to persist in water distribution systems provides a reservoir for food contamination, being directly by water contact or through contaminated food processing surfaces.

3.1.1. General characteristics of biofilms

In their natural habitats, bacteria grow preferentially as biofilm complex communities comprising single or multiple species, adhered to wet surfaces and embedded in a self-produced slimy matrix composed by Extracellular Polymeric Substances (EPS), such as polysaccharides, lipids, proteins, nucleic acids and enzymes (Lasa 2006; Brooks & Flint 2008; Shi & Zhu 2009; Simões et al. 2010).

Biofilm development is described as a five-stage process of adaptation and changing genetic regulation (Stoodley et al. 2002; Lasa 2006; Pereira da Silva et al. 2012): (1) initial attachment of cells to the surface; (2) production of the EPS; (3) early development of the biofilm; (4) maturation of the biofilm; (5) dispersion of bacterial cells from the biofilm (Figure 5).

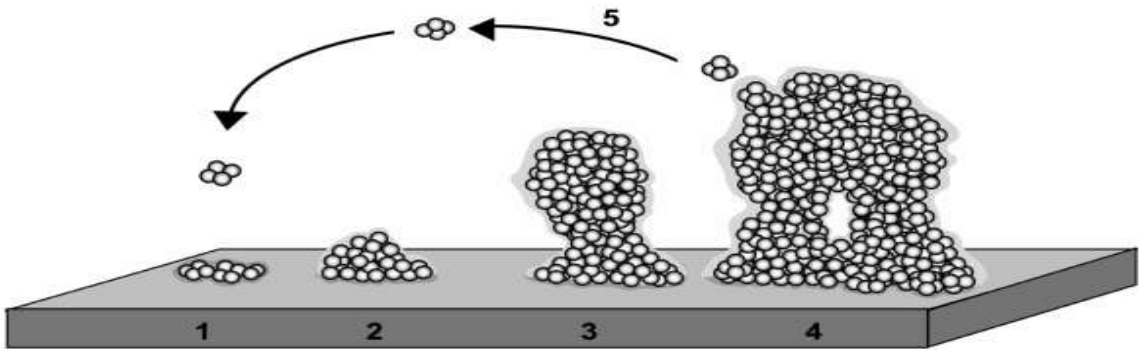

Figure 3 - Biofilm development as a five-stage process: (1) initial attachment of cells to the surface; (2) production of EPS; (3) early development of the biofilm; (4) maturation of the biofilm; (5) dispersion of bacterial cells from the biofilm (Extracted from Lasa, 2006)

(1) Initial attachment: Attachment is an initial weak interaction of bacteria with the substratum, involving Van der Waals and weak electrostatic forces and hydrophobic interactions (Chmielewski & Frank 2003; J. Brooks & Flint 2008; Pereira da Silva et al. 2012). Some components of the bacterial outer-membrane like flagella, fimbriae and lipopolysaccharides (LPS) confer hydrophilic properties to

the bacterial cells, making them more adhesive to hydrophilic materials (Chmielewski & Frank 2003; Shi & Zhu 2009). At this stage, adhesion to the surface is reversible and therefore many cells leave the surface, resuming to the planktonic lifestyle, while others continue the differentiation process leading to biofilm formation (Stoodley et al. 2002).

(2) Cellular proliferation and EPS production: After attachment to a solid surface, bacterial cells start producing EPS and adhesion becomes irreversible (Stoodley et al. 2002). Microcolony aggregation occurs through the aggregation of planktonic cells from the surrounding medium, as a result of quorum sensing (Chmielewski & Frank 2003)

(3) Development and (4) maturation of the biofilm: Maturation of the biofilm starts with the development of water channels and changing in cells physiology. As the biofilm matures, it become a highly organized system where water channels allow the exchange of nutrients and waste products with the surrounding medium (Stoodley et al. 2002; Shi & Zhu 2009).

(5) Detachment/cell dispersion: A reduction in EPS synthesis promotes the detachment of individual microcolonies that disperse and colonize other surfaces (Stoodley et al. 2002).

3.1.1.1. Factors influencing attachment and biofilm development

Biofilm formation is a complex process that relies on interactions between the bacterial cells, the surface and the surrounding medium (Shi & Zhu, 2009; Houdt & Michiels, 2010; Giaouris et al., 2013).

It has been observed that the extension of bacterial attachment correlates with surface roughness, and defects (J. Brooks & Flint 2008; Shi & Zhu 2009). Additionally, hydrophobicity of both cell membrane and adhesion surface is important in adhesion (M. Simões et al. 2010). The surface of most bacterial cells are negatively charged, which is adverse to bacterial adhesion however, the presence of flagella, fimbriae and lipopolysaccharides (LPS) conferrers hydrophobicity to bacterial cells, thus facilitating attachment and biofilm formation (Shi & Zhu 2009).

Environmental factors such as pH, temperature, osmolarity, oxygen levels, shear stress, nutrient availability and even the presence of other bacteria also plays an important role in initial attachment of bacterial cells to the surface (Shi & Zhu 2009; Giaouris et al. 2013).

3.1.1.2. Advantages of biofilm mode of growth

Biofilm formation confers fitness advantages to bacteria, representing their normal lifestyle in the environment. Bacterial biofilm formation could be driven by at least four values: (i) protection from stressful environmental conditions; (ii) competition for nutrients; (iii) benefits of metabolic interactions between microbial species, and (iv) acquisition of new adaptative phenotypic traits due to gene transfer. In fact, it is generally accepted and well documented that sessile cells can stand nutrient deprivation and are more resistant to shear forces, toxins, pH changes, host immune defences, antibiotics and sanitizers than their planktonic counterparts (Jefferson 2004; Giaouris et al. 2013).

The increased resistance of biofilm cells to antimicrobials can be explained by at least four different mechanisms: (i) a physical barrier form by the EPS matrix, limiting the diffusion of antimicrobials within the biofilm; (ii) resistance mechanisms, like detoxifying membrane transporters, that can be easily

horizontally transferred among biofilm cells; (iii) differentiation of bacterial cells into different physiological states, and (iv) less efficiency of the sanitizers by a modification of the environment (e.g. acidic pH) (Giaouris et al. 2013).

3.1.2. Biofilms in the food-industry

Adhesion of *Salmonella* to food surfaces was the first published report on foodborne bacterial biofilm. Since then, many reports documenting the persistence of foodborne pathogens like *Listeria monocytogenes, Yersinia enterocolitica*, *Campylobacter jejuni* and *Escherichia coli* O157:H7 on food and food contact surfaces associated with biofilm formation have been described (Shi & Zhu 2009) Biofilm formation is generally problematic to the food industry in what concerns to food quality and safety because the capacity of foodborne bacteria to survive stresses commonly encountered within food processing facilities (e.g. refrigeration, acidity, salinity and disinfection) is enhanced (Shi & Zhu 2009; Simões et al. 2010). Thus, biofilms formed in food-processing environments have the potential to act as a persistent source of microbial contamination and cross-contamination, increasing the food safety risk (Shi & Zhu, 2009; Giaouris et al., 2013).

In the food industry, processing equipments are constructed of different materials such as stainless steel (SS), glass, rubber, teflon, nylon and polytetrafluorothylene (PTFE). SS is regarded as an ideal material due to its physicochemical stability at various food-processing temperatures and high resistance to corrosion, however, studies have demonstrated that bacterial cells adhere preferentially to SS when compared to other metals, glass, rubber or PTFE (Sinde & Carballo 2000; Flint et al. 2000; Chmielewski & Frank 2003; Brooks & Flint 2008; Shi & Zhu 2009).

Biofilm control efforts most often on effective cleaning and disinfection procedures of potential control sites (Chmielewski & Frank 2003). These are distinct but complementary processes: cleaning is of utmost importance to improve the disinfection of the processing equipment (Simões et al. 2010). Cleaning processes can remove approximately 90% of the microorganisms associated with the surface but cannot be relied upon to kill them. Disinfectants are not formulated for cleaning purposes since they do not penetrate the biofilm matrix left on a surface after an ineffective cleaning procedure, and thus do not destroy all the biofilm living cells. Additionally, effectiveness of disinfectants is limited by the presence of soilless, water hardness, pH, temperature and surface properties (Chmielewski & Frank 2003; Simões et al. 2010). If sanitation procedures are ineffective, bacteria can redeposit at other locations and form biofilm. Once biofilms are allowed to form, sanitation procedures may not be fully effective in eliminating them (Chmielewski & Frank, 2003; Giaouris et al., 2013). Commercial disinfectants used in the food industry should be approved on the basis of European standard disinfection tests, thereby establishing an in-use concentration for the disinfectant for a specific contact time and temperature. Accordingly, the disinfectant should be able to demonstrate 5-log reduction in the suspension test for bactericidal efficacy (EN 1276) and 4-log reduction in the carrier test for bactericidal efficacy (EN 13697).

Biocides can be classified in at least 22 categories, on the basis of their functional chemical groups and/or into four groups, based on their action target: proteins, membrane, nucleic acids and cell wall; and the same biocide can have multiple targets within a bacterial cell (Gnanadhas et al. 2013).

Overall, the persistence of *Aeromonas* spp. in water distribution systems acts as a source of contamination, thus leading to a increasing need of control through effective cleaning and disinfection procedures in food processing facilities, which relies heavily on the use of disinfectants.
The present study aimed to evaluate the potential for biofilm formation, at both refrigeration and room temperatures, of representative members of an *Aeromonas* collection. It also regards the evaluation of chemical disinfection efficacy in removing aeromonads biofilms as well as in preventing biofilm formation.

3.2. Material and methods

3.2.1. Bacterial strains

For the biofilm assays, five aeromonads were chosen as representative of the overall collection (Table 7) based on their pathogenicity potential.

Table 7 – Representative aeromonads chosen for biofilm assays

Isolate	Species	Source	
A31	*Aeromonas* sp.	Portugal	Slaughterhouse-surface
A97	*Aeromonas* sp.	Portugal	Cheesemaking factory-surface
A172	*A.hydrophila* HG1	Bangladesh	Human stool
A259	*A.hydrophila*	Portugal	Clinical
S2	*Aeromonas* sp.	Portugal	Unchlorinated drinking water

Additional bacteria were included as positive controls of the assay: *A. hydrophila* (DSMZ 30187^T) and *Pseudomonas aeruginosa* (PAO1). All strains were stored at -80^{o}C in Brain Heart Infusion – BHI – broth (Scharlau, Barcelone, Spain) containing 20% ($^v/_v$) glycerol and routinely grown on BHI agar at 30^{o}C.

3.2.2. Preparation of the inoculums

An overnight culture of each isolate was grown in 10 mL of BHI broth at 30^{o}C. The optical density (OD) was measured at 600 nm to ensure inoculums with a cellular concentration of 10^{-7} CFU/mL. The cells in the corresponding volume were washed by centrifugation for 10 min at 14000 rpm. The supernatant was discarded, the pellet resuspended in 1 mL of Phosphate Buffer Saline (PBS) 0.1 M (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4 and 0.24 g/L KH_2PO_4) and washed by centrifugation for 10 min at 14000 rpm. Lastly, the cells were resuspended in a volume of 200 µL of PBS.

The subsequent assays included three replicates of the same bacterium (technical replicates) and were repeated three times using independent bacterial cultures (biological replicates).
Plates were organized as shown in Figure 5. The sterility control of the assays consisted in non-inoculated wells (C-).

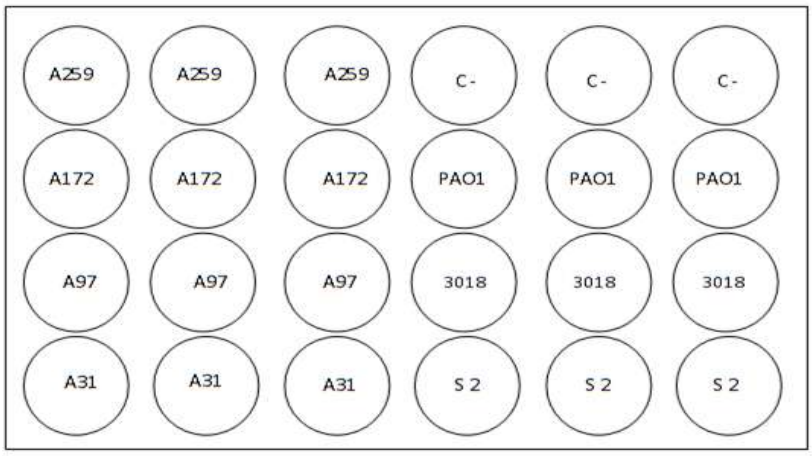

Figure 4 - Representative scheme of replicates organization in each plate.

3.2.3. Biofilm formation on stainless steel coupons

To obtain a final concentration of 10^{-8} CFU/mL, 100 µL of each inoculum were added in triplicate to the wells of two sterile 24-well microtitre-plates containing 900 µL of BHI and a stainless steel (SS) coupon (11 mm X 2 mm) (Figure 7). Plates were incubated at both 4°C and 20°C, for 48 h.

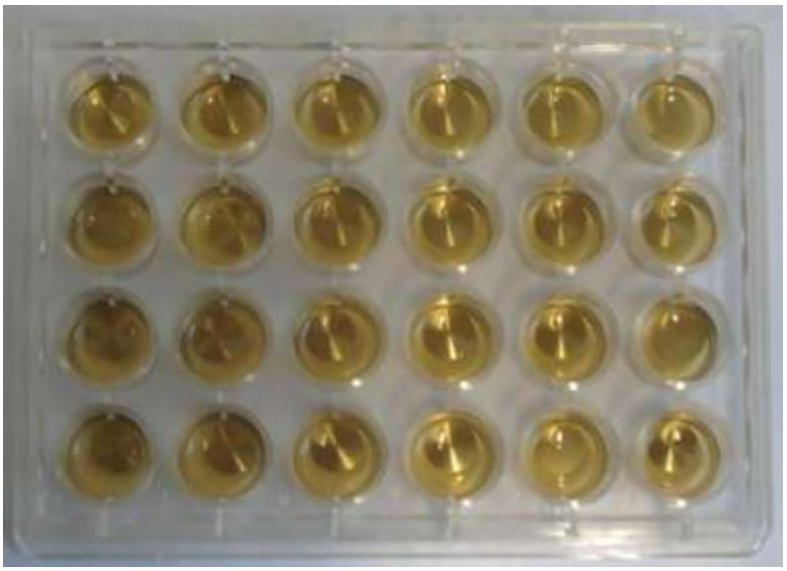

Figure 5 - Representative scheme of SS coupons within the wells of a microtitre plate.

Biofilm quantification

After incubation time, SS coupons were carefully removed from the plate with a tweezer. In order to detach non-adherent or loosely cells (Fonseca et al. 2001), SS coupons were rinsed with 3 mL of PBS 0,1 M as represented in Figure 8.

Figure 6 - SS coupons washing procedure

Then, each SS coupon was placed in a new 24-well microtitre-plate containing 1 mL of PBS 0.1 M 0.1% Tween 80 (v/v) (Extremina et al. 2011) and plates were sonicated for 30 min in an ultrasonic cleaning bath. Then, according to Chen et al. 2003, 250 µL of each sample were loaded into the first well of a 96-well microtitre-plate and tenfold serial dilutions were made by transferring 25 µL from the first well into 225 µL of medium in the second well, mixing 10 times and repeating the process. Thereafter, according to the Miles & Misra (1938) technique, three replicates of a 10 µL drop of each dilution for each sample were plated on BHI (Figure 9), followed by overnight incubation at 30^{o}C. After bacterial growth, the number of adherent bacteria colonies was assessed.

Figure 7 - Three replicates of a 10 µL drop of six tenfold serial dilutions, plated on BHI.

3.2.4. Effects of disinfectants on biofilms

The efficacy of three different commercial disinfectants used in the food industry in removing aeromonads biofilm/preventing biofilm formation on SS coupons was assessed. The specifications of each detergent are listed in Table 8.

Table 8 - Specifications of the commercial disinfectants used in this study

Disinfectant (*)	Composition	Class		Standardization	
		Functional group	Action targets	EN 1276	EN 13697
Suredis®	*amphoteric surfactants*	Surface active agents	Cell membrane	1%, 5 min, 20°C	1%, 5 min, 23°C
(A)	*diamines*		Nucleic acid		
Deogen®	sodium hypochlorite	Chlorine compounds	Proteins	0.75%, 5 min, 20°C	not specified
(B)	sodium hydroxid		Nucleic acids		
Divosan Activ®	peracetic acid (5%)	Peroxygens	Cell membrane	0.1%, 5 min, 20°C	not specified
(C)	hydrogen peroxid		Proteins		
			Nucleic acids		

(*) detergents were gently provided by Diversey Portugal, Unipessoal, Sintra, Portugal.

3.2.4.1. Biofilm eradication

After biofilm formation, the SS coupons were carefully removed from the plate and rinsed with 3 mL of PBS 0,1 M**,** in order to detach non-adherent cells (Fonseca et al. 2001). Thereafter, the SS coupons were individually immersed, for 5 min, in 1 mL of each disinfectant at the recommended concentration (Suredis® 1%, Deogen® 0.75% and Divisan Activ® 0.1%). After disinfection time (5 min, 20°C), quantification of the remaining biofilm was performed as described in 3.2.3.1.

3.2.4.2. Inhibition of biofilm formation

To obtain a final concentration of 10^{-8} CFU/mL, 100 µL of each inoculum were added in triplicate to the wells of a sterile 24-well microtitre-plate containing a mixture of 900 µL of BHI containing each disinfectant diluted at the recommended concentration and a stainless steel (SS) coupon. The plates were incubated at 20°C, for 48 h. Quantification of the remaining biofilm was performed as described in 3.2.3.1. Decimal log reduction was calculated using the equation: $LgR = \lg N_0 - \lg N_a$ (R – reduction; N_0 – number of cells per mL in the biofilm; N_0 – number of surviving cells per mL).

3.3. Results and discussion

In the present study, we aimed to evaluate *Aeromonas* spp. biofilm forming ability in SS coupons at both refrigeration (4°C) and room (20°C) temperatures, after 48 h of incubation.

In a first approach, the collection of 118 *Aeromonas* was submitted to an alternative faster screening technique that would possible allow to choose representative aeromonads for the subsequent biofilm assays, namely the Congo red agar method. This method, described by Freeman et al. (1989), is based on bacterial morphological changes after incubation in a medium composed by BHI (Scharlau, Barcelone, Spain) 37 g/L, agar (Scharlau, Barcelone, Spain) 7 g/L, sucrose 50 g/L and Congo red stain (0.8 g/L) and incubated for 48 h, at 30°C, for 48 h. After bacterial growth, slime production is detected by the presence of dark black colonies. Arciola et al. 2002 revised this method using a six colors based scale to determine whether staphylococcal strains were slime producers or not: *very red, red, light red, light black, black* and *very black*.

Aeromonads morphological changes after growth in BHI-Congo red medium, for 48h at 30°C, were evaluated independently by the present author and a colleague (data not shown). Results based on the six color scale were subjective since it gave origin to different interpretations. This method was not suitable for aeromonads selection for biofilm assays, however it allowed to presumptively consider at least 40 (33,9%) isolates as slime producers. Hence, representative aeromonads were chosen regarding their source of origin and pathogenicity potential.

Preliminary studies comparing (i) 24 and 48 h of incubation led to the selection of a 48 h period for subsequent assays (data not shown) and (ii) five, 10, 15, 20 and 30 min of sonication, led to the selection of 30 min (data not shown). Results obtained are illustrated in Figure 10.

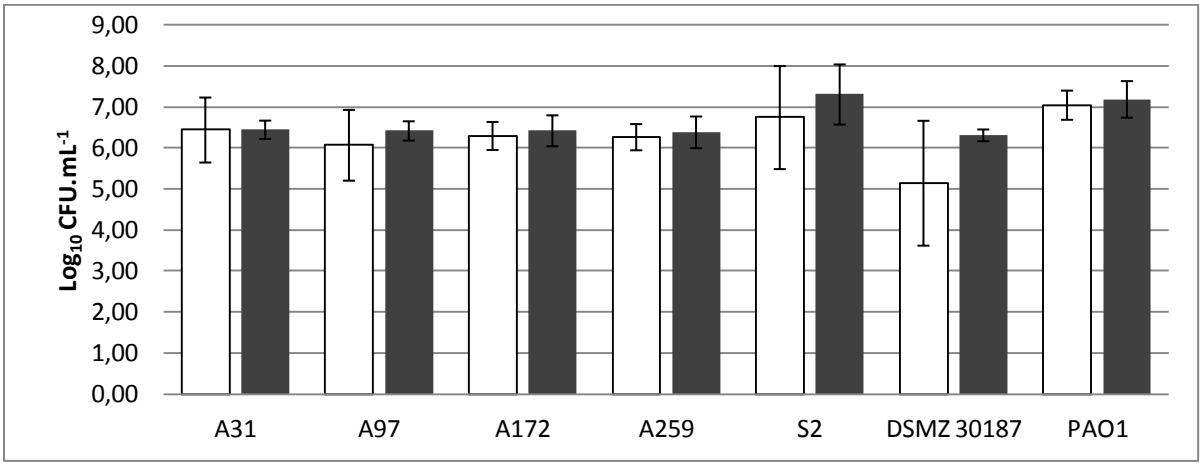

Figure 8 - Biofilm formation on SS coupons by five aeromonads, A. hydrophila subs. hydrophila (DSMZ 30187T) and Pseudomonas aeruginosa (PAO1), after 48 h of incubation at both 4°C (□) and 20°C (■). The error bars represent the mean values ± standard deviations.

All isolates under analysis were able to form biofilm. Biofilm formation at 4°C ranged values between 10^5 CFU.mL^{-1} (DSMZ 30187) and 10^7 CFU.mL^{-1} (PAO1) while biofilm formation at 20°C ranged between 10^6 CFU.mL^{-1} (DSMZ 30187) and 10^7 CFU.mL^{-1} (PAO1). We found no significant differences between temperatures or isolates. However, isolate S2 seemed to possess higher ability to form biofilm. Although, not as strong as *Pseudomonas aeruginosa* (PAO1).

These results might be of special concern regarding aeromonads source of origin, especially in what concerns isolates A31, A97 and S2, since A31 and 97 were isolated from food processing facilities, namely a slaughterhouse and a cheesemaking factory and S2 was isolated from superficial waters from Rio Tejo. Results obtained indicate that isolates A31 and A97 have the ability to persist as biofilm communities in the places of isolation, constituting source of food contamination. Regarding isolate S2, if able to resist water chlorination, it should be capable of persisting in water systems and thus colonize and form biofilm in food contact surfaces, being a possible source of food contamination.

In this investigation, we also aimed to assess the efficacy of three different commercial disinfectants in removing sessile aeromonads from SS coupons. To determine whether the three disinfectants were effective or not, we considered a 4-log reduction. According to the specifications, bactericidal activity of disinfectant A was tested for both standards EN 1276 and EN 13697, meaning that it should be able to demonstrate a 5-log reduction for planktonic cells and a 4-log for sessile cells, within 5 min of contact; disinfectants B and C were tested for EN 1276 , meaning that they should demonstrate a log-reduction of 4-log for sessile cells but there are no information regarding EN 13697. Thus, a 4-log reduction was chosen. Results are presented in Table 9.

Table 9 – Decimal log reduction (lg R) achieved by the commercial disinfectants

	lg R (A)		lg R (B)		lg R (C)	
Isolates	4°C	20°C	4°C	20°C	4°C	20°C
A31	2	4	6	5	6	6
A97	4	4	5	5	5	6
A172	4	5	6	5	5	6
A259	5	6	6	6	5	6
S2	3	4	6	6	6	6
DSM30187^T	5	5	4	5	4	6
PAO1	4	4	7	6	6	4
Average $^+$	4	5	6	5	5	6
Average ^{x}	4		6		6	

(*) Average log reduction per disinfectant and temperature

(+) Average log reduction per disinfectant

Even though disinfectants B and C were not tested according to EN 13697 or failed to pass through the criteria of this standard, in our study, they demonstrated an average reduction of 6-log in biofilm removal. Contrarily, disinfectant A, which passed through the EN 13697, had an average reduction of only 4-log instead of 5-log. However, since we considered 4-log reduction as criteria, the overall results of the three commercial disinfectants were satisfactory.

Additionally, disinfectant A were not effective in removing biofilms of aeromonads A31 and S2, formed at 4ºC (2 and 3-log reduction, respectively), which is of special concern regarding their source of origin (slaughterhouse and residual waters). The inability of this disinfectant in removing biofilms formed by these isolates may allow them to persist as a source of food contamination even after disinfection procedures.

We also observed that disinfectants A and C had lower efficacy at 4ºC (4 and 5-log reduction, respectively), however this might be expectable since disinfectants efficacy augments with increasing temperatures (Baptista 2003). Contrarily, a lower temperature seemed to enhance disinfectant B efficacy, which presented reductions of 6-log at 4°C and 5-log at 20°C. These results lead to the assumption that, in real context, refrigerators disinfection should be undertaken using disinfectant B instead of disinfectants A and C.

Nevertheless, overall the results demonstrated that all three commercial disinfectants were efficient in removing sessile cells of aeromonads from SS coupons at both 4ºC and 20ºC.

In order to evaluate whether these commercial disinfectants were effective in inhibiting biofilm formation, aeromonads were left to attach to SS coupons, at 20°C, in the presence of each disinfectant. Results are presented in Figure 11.

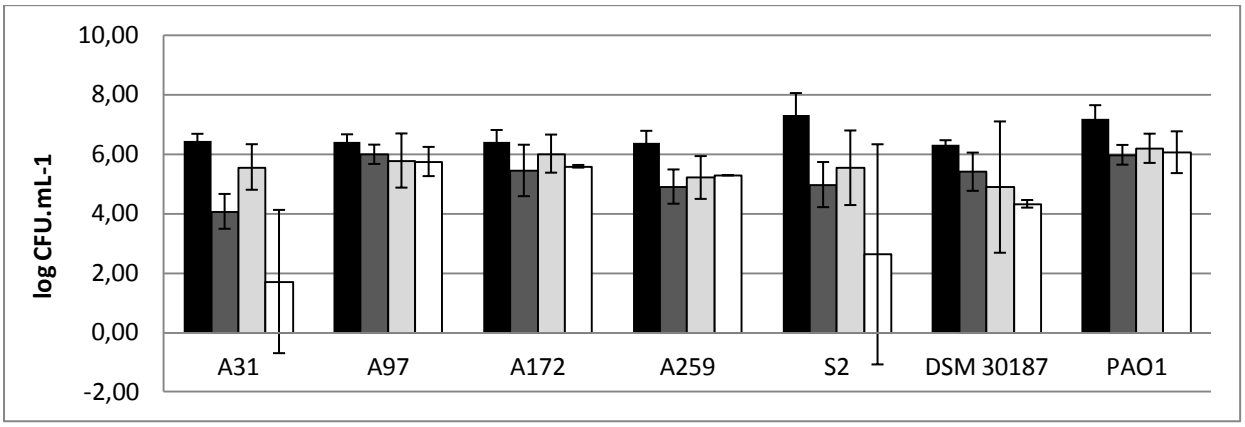

Figure 9 - Inibition of biofilm formation on stainless steel coupons by the five aeromonads, DSMZ 30187^T and *Pseudomonas aeruginosa* PAO1, after 48 h of incubation at 20°C. The error bars represent the mean values.
( ) Initial biofilm formation; ( ) Disinfectant A; ( ) Disinfectant B; ( ) Infectant C.

We observed that none of the disinfectants was totally efficient in inhibiting biofilm formation. Disinfectant C showed higher effectiveness in preventing biofilm formation, especially in what concerns to aeromonads A31 and S2, which correlates well with previous results.

Such results might indicate that, even in the presence of residual disinfectant concentrations, planktonic aeromonads will be able to survive and form biofilms. Moreover, we should have in consideration that, as the surfaces dry after disinfection procedures, disinfectants form a pellicle that will be an excellent culture medium for microorganism's growth (Baptista 2003). Additionally, the presence of soiling is not only a nutritive support for microorganisms but it also reacts with disinfectants, neutralizing them and consequently reducing their effectiveness. Standard soiling conditions using 3 g/L bovine albumin solution were not tested in the present investigation, however BHI broth is a nutritive medium that possible had the same effect on both microorganisms and disinfectants. These results highlighted how important it is to respect all cleaning procedures before disinfection.

Quality of biofilm assays depends on its reproducibility; however the development of biofilms is a stochastic process and one of the major obstacles in biofilms quantification is that they are very difficult to reproduce (Heydorn et al. 2000).
At the present investigation, technical and biological replicates were undertaken. The final CFU value was obtained by calculating the average CFU of between replicates. Additionally, the respective standard deviations were also calculated. However, no statistical analysis could be applied because of the heterocedasticity found among the results obtained.
In the present study, SS coupons washing procedure seemed to be the major cause of this problem. Although this procedure has been carried out carefully, it could be possible that some sessile cells may have been swept away by water. To overcome this problem, another type of procedure should be tested, like dipping the coupons in 24 well microtitre plates. However, it is not possible to perform this procedure in the first place. Additionally, more replicates could be made to achieve more reliable results.

3.4. Conclusions

In the present study we aimed to (i) evaluate the biofilm forming ability of five representative aeromonads of a collection of 118 *Aeromonas,* at both 4^{o}C and 20^{o}C, and (ii) assess the efficacy of three commercial disinfectants in removing preformed biofilms, as well as in preventing biofilm formation.
All *Aeromonas* evaluated were capable of biofilm formation and no significant differences were observed between isolates or temperatures.
In what concerns disinfectants efficacy, all products under analysis were efficient in removing aeromonads biofilms from SS coupons; however, they were not capable of preventing biofilm formation.

Chapter 4 – Final considerations

The present study had two principal objectives: assessment of the genetic diversity of a collection of *Aeromonas* isolated from different sources of origin and the evaluation of the biofilm forming ability in SS coupons by selected strains of the collection. Briefly, the following conclusions were achieved:

- A high genetic diversity among the analyzed isolates was found, as expected, as this is a characteristic of the genus *Aeromonas;*

- The multilocus sequence analysis of six housekeeping genes (*gyrB, gltA, groL, metG, ppsA* and *recA*) helped to clarify some taxonomical issues regarding aeromonads species allocation;

- All representative aeromonads analyzed were capable of biofilm formation in SS coupons at both room and refrigeration temperatures;

- Commercial disinfectants analyzed were efficient in removing preformed biofilm in SS coupons, at both room and refrigeration temperatures; however they were not efficient in preventing biofilm formation.

Results obtained at the present study should be checked against previous results corresponding to aeromonads pathogenicity potential, namely antibiotic resistance and virulence profiles, and adhesion to caco-2 mammalian cells and their invasion.

To reduce or eliminate microorganisms found on food contact surfaces, cleaning and disinfection procedures have been extensively used over the years. However, once formed, biofilms are known for their resistance to antimicrobial agents and may be hard to eradicate, persisting as potential food contaminants. Thus, potential control strategies preventing bacterial adhesion and biofilm formation (e.g. enzymes, quorum sensing inhibitors, bacteriocins, phages, nanoemulsions, surfactants) should be regarded (Simões M., Simões L. 2010; Giaouris et al. 2013).The improved understanding of the physiology of aeromonads biofilms will probably facilitate the methods for controlling their biofilms in food areas.

Hence, further studies should be undertaken, especially regarding aeromonads biofilm forming ability, in order to simulate several other conditions encountered in real food processing systems. These might include:

- Different pH values – *Aeromonas* can growth with pH values between 4.5 and 9.0, which means that they are able to grow in a great variety of food; thus, it is important to assess their biofilm forming ability at different pH values;

- Different NaCl concentrations – *Aeromonas* can growth at NaCl concentrations ranging values between 0 to 4%; the addition of NaCl a widely used food preservation method; thus it is important to evaluate the biofilm forming ability at different NaCl concentrations;

- Multi-species biofilm with other bacteria (*Pseudomonas* spp. and/or *Salmonella* spp., for example) – in food processing environments a variety of bacteria can be part of a biofilm community; additionally, several studies indicate that attachment might increase when bacteria grows in multi-species biofilms rather than single-species biofilms;

- Utilization of meat, fish or milk extract as growing media – alone or combined with at least one of the conditions described above, it should simulate better real biofilm formation on food.

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Appendix A

Table 10 - PCR amplifications and complete target sequences of the complete *Aeromonas* spp. collection

	Species	Geographical source	Biological source	PCR amplification						Complete target sequence					
				metG	*ppsA*	*groL*	*gltA*	*gyrB*	*recA*	*metG*	*ppsA*	*groL*	*gltA*	*gyrB*	*recA*
DSMZ 13956	*A.bestiarium*		Infected fish	+	+	+	+	+	+	-	-	-	-	-	-
DSMZ 6394	*A.enteropelogenes*		Human faeces	+	+	+	+	+	+	-	-	-	-	-	-
DSMZ 7386	*A.veronii*		Sputum of drowning victim	+	+	+	+	+	+	-	-	-	-	-	-
DSMZ 7323	*A.caviae*			+	+	+	+	+	+	-	-	-	+	+	+
DSMZ 30187	*A.hydrophyla sub hydrophila*		Milk	+	+	+	+	+	+	-	-	-	-	+	-
DSMZ 4882	*A.shubertii*		Forehead abscess	+	+	+	+	+	+	-	-	-	+	-	+
A154	*A.caviae HG4*	Bangladesh	Human faeces	+	+	+	+	+	+	+	-	+	+	+	+
A157	*A.caviae HG4*	Bangladesh	Human faeces	+	+	+	+	+	+	+	+	+	+	+	+
A163	*A.hydrophila HG1*	Bangladesh	Human faeces	+	+	+	+	+	+	+	+	+	+	+	+
A172	*A.hydrophila HG1*	Bangladesh	Human faeces	+	+	+	+	+	+	+	+	+	+	+	+
A174	*A.hydrophila HG1*	Bangladesh	Human faeces	+	+	+	+	+	+	+	+	+	-	+	+
A143	*A.caviae/media HG 5B*	Belgium	Clinical	+	+	+	+	+	+	+	+	+	+	+	+
A147	*A.caviae HG4*	Belgium	Clinical	+	+	+	+	+	+	+	-	+	+	+	+
A150	*A.caviae HG4*	Belgium	Clinical	+	+	+	+	+	+	+	+	+	+	+	+
A160	*A.caviae HG4*	Belgium	Drinking water	+	+	+	+	+	+	-	-	+	+	+	-
A161	*A.hydrophila HG3*	Belgium	Clinical	+	+	+	+	+	+	+	+	+	+	+	+
A178	*A.hydrophila HG2*	Belgium	Food-vegetables	-	-	-	-	-	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
A179	*A.hydrophila HG3*	Belgium	Drinking water	-	-	-	-	+	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
A184	*A.hydrophila HG3*	Belgium	Drinking water	+	+	+	+	+	+	+	-	+	+	+	+
A186	*A.hydrophila HG3*	Belgium	Drinking water	+	+	+	+	+	+	+	+	+	+	+	+
A188	*A.hydrophila HG3*	Belgium	Food-meat	+	+	+	+	+	+	+	+	+	+	+	+
A193	*A.hydrophila HG3*	Belgium	Fish	+	+	+	+	+	+	+	+	+	+	+	+
A194	*A.hydrophila HG3*	Belgium	Fish	+	+	+	+	+	+	-	-	-	+	+	+
A195	*A.hydrophila HG3*	Belgium	Meat	+	+	+	+	+	+	+	-	+	+	+	+

	Species	Geographical source	Biological source	PCR amplification						Complete target sequence					
				metG	*ppsA*	*groL*	*gltA*	*gyrB*	*recA*	*metG*	*ppsA*	*groL*	*gltA*	*gyrB*	*recA*
A200	*A.hydrophila HG3*	Belgium	Fish	+	+	+	+	+	+	+	+	+	+	+	+
A202	*A.veronii*	Belgium	Fish	+	+	+	+	+	+	+	+	+	+	+	+
A206	*A.veronii HG8*	Belgium	Clinical	+	+	+	+	+	+	-	+	+	+	+	+
A208	*A.veronii HG8 / 10*	Belgium	Drinking water	+	+	+	+	+	+	+	-	-		-	-
A210	*A.veronii HG8 / 10*	Belgium	Drinking water	+	+	+	+	+	+	+	+	-	+	+	+
A230	*A.caviae*	Brasil	Clinical	+	+	+	+	+	+	+	+	+	+	+	+
A231	*A.trota*	Brasil	Clinical	+	+	+	+	+	+	+	-	+	+	+	+
A232	*A.hydrophila*	Brasil	Clinical	+	+	+	+	+	+	+	+	+	+	+	+
A234	*A.jandaei*	Brasil	Clinical	+	+	+	+	+	+	+	-	-	-	+	-
A236	*A.veronii*	Brasil	Clinical	+	+	+	+	+	+	+	+	+	+	+	+
A237	*A.hydrophila*	Brasil	Clinical	+	+	+	+	+	+	+	-	+	+	+	+
A239	*A.veronii*	Brasil	Clinical	+	+	+	+	+	+	+	-	-	+	+	+
A241	*A.trota*	Brasil	Clinical	+	+	+	+	+	+	+	-	+	+	+	-
A244	*A.veronii*	Brasil	Clinical	+	+	+	+	+	+	+	+	-	+	+	+
A245	*A.veronii*	Denmark	Clinical	+	+	+	+	+	+	+	+	+	+	+	+
A246	*A.veronii*	Denmark	Clinical	+	+	+	+	+	+	+	-	-	+	-	-
A248	*A.bestiarum*	Denmark	Clinical	+	+	+	+	+	+	+	+	-	+	+	+
A249	*A.hydrophila*	Denmark	Clinical	+	+	+	+	+	+	-	-	-	-	+	-
A252	*A.media*	Denmark	Clinical	+	+	+	+	+	+	+	+	+	+	+	+
A253	*A.punctata*	Denmark	Clinical	+	+	+	+	+	+	+	+	+	+	+	+
A1	*Aeromonas* sp.	Portugal	Slaughterhouse-surface	+	+	+	+	+	+	+	-	+	+	+	-
A3	*Aeromonas* sp.	Portugal	Slaughterhouse-surface	+	+	+	+	+	+	+	-	+	+	+	+
A4	*Aeromonas* sp.	Portugal	Slaughterhouse-surface	+	+	+	+	+	+	+	-	+	+	+	+
A5	*Aeromonas* sp.	Portugal	Slaughterhouse-surface	+	+	+	+	+	+	+	+	+	+	+	+
A6	*Aeromonas* sp.	Portugal	Slaughterhouse-surface	+	+	+	+	+	+	+	-	+	+	+	+
A7	*Aeromonas* sp.	Portugal	Slaughterhouse-surface	+	+	+	+	+	+	+	-	+	+	+	+
A8	*Aeromonas* sp.	Portugal	Slaughterhouse-surface	+	+	+	+	+	+	+	-	+	+	+	+
A11	*Aeromonas* sp.	Portugal	Slaughterhouse-surface	+	+	+	+	+	+	+	+	+	+	+	+
A13	*Aeromonas* sp.	Portugal	Slaughterhouse-surface	+	+	+	+	+	+	+	-	+	+	+	+
A17	*Aeromonas* sp.	Portugal	Slaughterhouse-surface	+	+	+	+	+	+	+	-	+	+	-	-
A23	*Aeromonas* sp.	Portugal	Slaughterhouse-surface	+	+	+	+	+	+	+	+	+	+	+	+
A24	*Aeromonas* sp.	Portugal	Slaughterhouse-surface	+	+	+	+	+	+	+	+	+	+	+	+
A25	*Aeromonas* sp.	Portugal	Slaughterhouse-surface	+	+	+	+	+	+	+	+	+	+	+	+

	Species	Geographical source	Biological source	PCR amplification						Complete target sequence					
				metG	*ppsA*	*groL*	*gltA*	*gyrB*	*recA*	*metG*	*ppsA*	*groL*	*gltA*	*gyrB*	*recA*
A26	*Aeromonas* sp.	Portugal	Slaughterhouse-surface	+	+	+	+	+	+	+	-	+	+	+	+
A27	*Aeromonas* sp.	Portugal	Slaughterhouse-surface	+	+	+	+	+	+	+	+	+	+	+	+
A28	*Aeromonas* sp.	Portugal	Slaughterhouse-surface	+	+	+	+	+	+	+	+	+	+	+	+
A31	*Aeromonas* sp.	Portugal	Slaughterhouse-surface	+	+	+	+	+	+	+	+	+	+	+	+
A33	*Aeromonas* sp.	Portugal	Slaughterhouse-surface	-	+	-	-	-	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
A37	*Aeromonas* sp.	Portugal	Slaughterhouse-surface	+	+	+	+	+	+	+	-	+	+	+	-
A38	*Aeromonas* sp.	Portugal	Slaughterhouse-surface	+	+	+	+	+	+	+	-	-	-	-	-
A42	*Aeromonas* sp.	Portugal	Supermarket-surface	+	+	+	+	+	+	+	+	+	+	+	-
A44	*Aeromonas* sp.	Portugal	Supermarket-surface	+	+	+	+	+	+	+	-	-	-	-	+
A50	*Aeromonas* sp.	Portugal	Supermarket-surface	+	+	+	+	+	+	+	-	-	+	-	+
A52	*Aeromonas* sp.	Portugal	Supermarket-surface	+	+	+	+	+	+	+	-	+	+	+	+
A53	*Aeromonas* sp.	Portugal	Supermarket-surface	+	+	+	+	+	+	+	+	+	+	+	+
A57	*Aeromonas* sp.	Portugal	Supermarket-surface	+	+	+	+	+	+	+	+	+	+	+	+
A61	*Aeromonas* sp.	Portugal	Supermarket-surface	+	+	+	+	+	+	+	+	+	+	+	+
A62	*Aeromonas* sp.	Portugal	Supermarket-surface	+	+	+	+	+	+	+	-	+	+	+	+
A68	*Aeromonas* sp.	Portugal	Supermarket-surface	+	+	+	+	+	+	+	+	+	+	-	+
A73	*Aeromonas* sp.	Portugal	Supermarket-fish	+	+	+	+	+	+	+	+	+	+	-	+
A75	*Aeromonas* sp.	Portugal	Supermarket-fish	+	-	+	+	+	+	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
A76	*Aeromonas* sp.	Portugal	Supermarket-fish	+	-	+	+	+	+	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
A77	*Aeromonas* sp.	Portugal	Supermarket-meat	+	+	+	+	+	+	+	+	+	+	+	+
A78	*Aeromonas* sp.	Portugal	Supermarket-meat	+	+	+	+	+	+	+	+	+	+	+	+
A85	*Aeromonas* sp.	Portugal	Supermarket-surface	+	+	+	+	+	+	+	-	+	+	+	+
A92	*Aeromonas* sp.	Portugal	Supermarket-surface	+	+	+	+	+	+	+	+	+	+	+	+
A93	*Aeromonas* sp.	Portugal	Supermarket-surface	+	+	+	+	+	+	+	+	+	+	+	+
A94	*Aeromonas* sp.	Portugal	Supermarket-surface	+	+	+	+	+	+	+	+	+	+	+	+
A95	*Aeromonas* sp.	Portugal	Supermarket-surface	+	+	+	+	+	+	+	+	+	+	+	+
A97	*Aeromonas* sp.	Portugal	Cheesemaking factory-surface	+	+	+	+	+	+	+	+	+	+	+	+
A98	*Aeromonas* sp.	Portugal	Cheesemaking factory-surface	+	+	+	+	+	+	+	+	+	+	+	+
A99	*Aeromonas* sp.	Portugal	Cheesemaking factory-surface	+	+	+	+	+	+	+	+	+	+	+	+
A100	*Aeromonas* sp.	Portugal	Cheesemaking factory-surface	+	+	+	+	+	+	+	+	+	+	-	+
A101	*Aeromonas* sp.	Portugal	Cheesemaking factory-surface	+	+	+	+	+	+	+	+	+	+	+	+
A102	*Aeromonas* sp.	Portugal	Cheesemaking factory-surface	+	+	+	+	+	+	+	+	-	+	+	+
A104	*Aeromonas* sp.	Portugal	Cheesemaking factory-surface	+	+	+	+	+	+	+	+	+	+	+	+

	Species	Geographical source	Biological source	PCR amplification						Complete target sequence					
				metG	*ppsA*	*groL*	*gltA*	*gyrB*	*recA*	*metG*	*ppsA*	*groL*	*gltA*	*gyrB*	*recA*
A105	*Aeromonas* sp.	Portugal	Cheesemaking factory-water	+	+	+	+	+	+	+	+	+	+	+	+
A108	*Aeromonas* sp.	Portugal	Vegetables	-	-	-	-	-	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
A116	*Aeromonas* sp.	Portugal	Vegetables	-	-	+	-	-	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
A123	*Aeromonas* sp.	Portugal	Vegetables	-	-	-	-	+	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
A127	*Aeromonas* sp.	Portugal	Vegetables	+	+	+	+	+	+	-	-	-	-	+	-
A255	*A.hydrophila*	Portugal	Clinical	+	+	+	+	+	+	+	+	+	+	+	+
A256	*A.hydrophila*	Portugal	Clinical	+	-	+	+	+	+	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
A257	*A.hydrophila*	Portugal	Clinical	+	-	+	+	+	+	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
A258	*A.hydrophila*	Portugal	Clinical	+	+	+	+	+	+	+	+	+	+	+	+
A259	*A.hydrophila*	Portugal	Clinical	+	+	+	+	+	+	+	+	+	+	+	+
A260	*Aeromonas* sp.	Portugal	Clinical	-	-	-	+	+	+	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S1	*Aeromonas* sp.	Portugal	Unchlorinated drinking water	+	+	+	+	+	+	+	+	+	+	+	+
S2	*Aeromonas* sp.	Portugal	Unchlorinated drinking water	+	+	+	+	+	+	+	-	-	+	+	+
S3	*Aeromonas* sp.	Portugal	Unchlorinated drinking water	+	+	+	+	+	+	+	-	+	+	+	+
S4	*Aeromonas sp.*	Portugal	Unchlorinated drinking water	+	+	+	+	+	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S5	*Aeromonas* sp.	Portugal	Unchlorinated drinking water	+	+	+	+	+	+	+	-	+	+	+	+
S6	*Aeromonas* sp.	Portugal	Unchlorinated drinking water	+	+	+	+	+	+	+	+	+	+	+	+
S7	*Aeromonas* sp.	Portugal	Unchlorinated drinking water	+	+	+	+	+	+	+	-	-	-	-	-
S8	*Aeromonas* sp.	Portugal	Unchlorinated drinking water	+	+	+	+	+	+	+	-	+	+	-	+
S10	*Aeromonas* sp.	Portugal	Unchlorinated drinking water	+	+	+	+	+	+	+	+	+	+	+	+
S13	*Aeromonas* sp.	Portugal	Unchlorinated drinking water	+	+	+	+	+	+	+	+	+	+	+	+
S15	*Aeromonas* sp.	Portugal	Unchlorinated drinking water	-	-	-	-	-	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S17	*Aeromonas* sp.	Portugal	Unchlorinated drinking water	-	-	-	-	-	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S18	*Aeromonas* sp.	Portugal	Unchlorinated drinking water	+	+	+	+	+	+	+	-	-	-	+	+
A219	*A.caviae HG4*	Thailand	Stool	+	+	+	+	+	+	+	+	+	+	+	+
A220	*A.caviae HG4*	Vietnam	Fish	+	+	+	+	+	+	+	+	+	+	+	-
A222	*A.caviae HG4*	Vietnam	Water	+	+	+	+	+	+	+	-	+	-	+	+
A226	*A.caviae HG4*	Vietnam	Stool	+	+	+	+	+	+	+	+	+	+	+	+

Isolates from Belgium, Bangladesh, Thailand and Vietnam were kindly provided by Professor Geert Huys, PhD, Faculty of Sciences, Ghent University, Belgium

Clinical strains from Portugal were kindly provided by Dra. Joana Selada, Hospital de Cascais Dr. Joaquim de Almeida

Appendix B

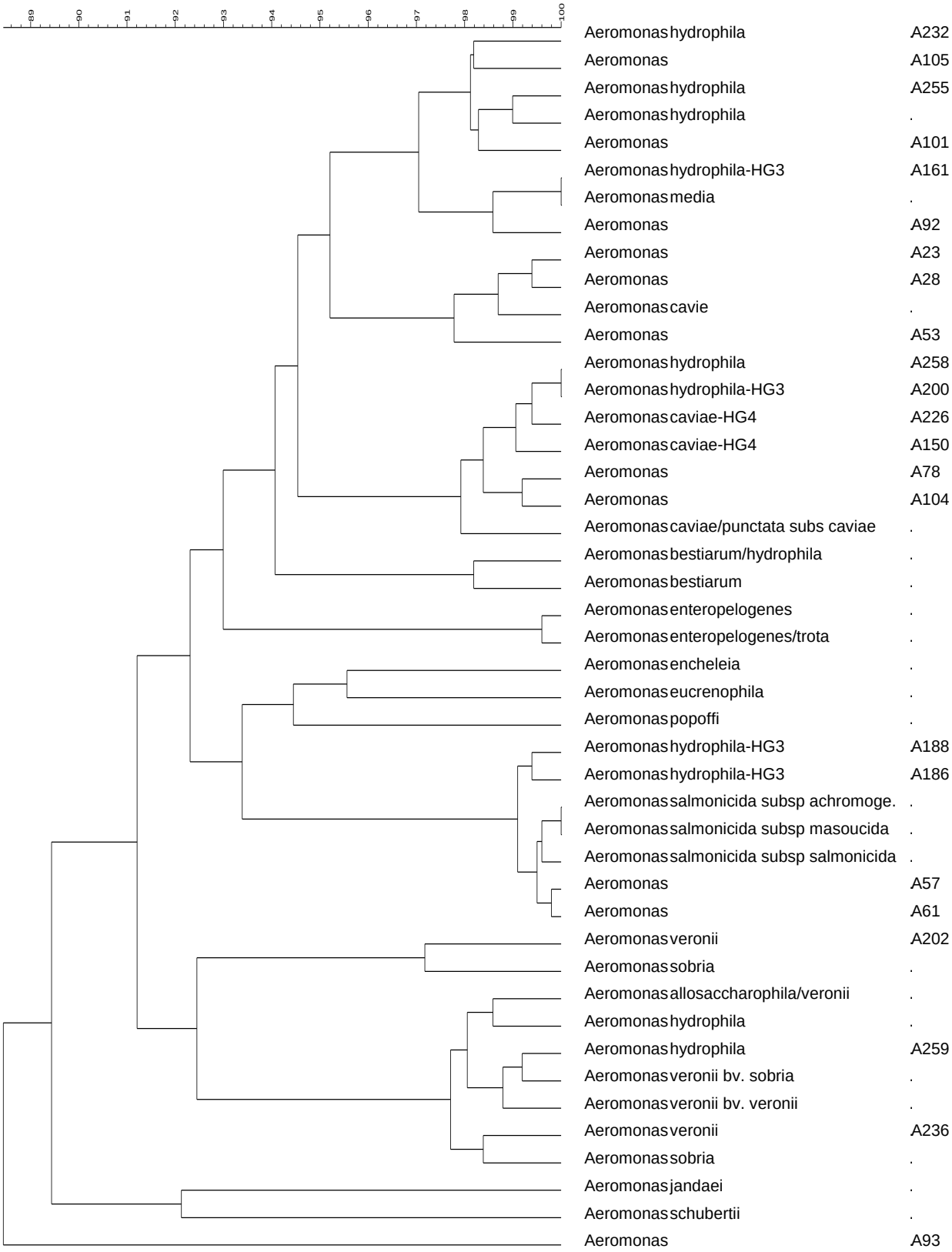

Figure 10 – Dendrogram obtained by multiple alignment of the concatenated sequences of *gltA* gene and grouped by the agglomerative clustering of unweighted pair group method with arithmetic mean (UPGMA).

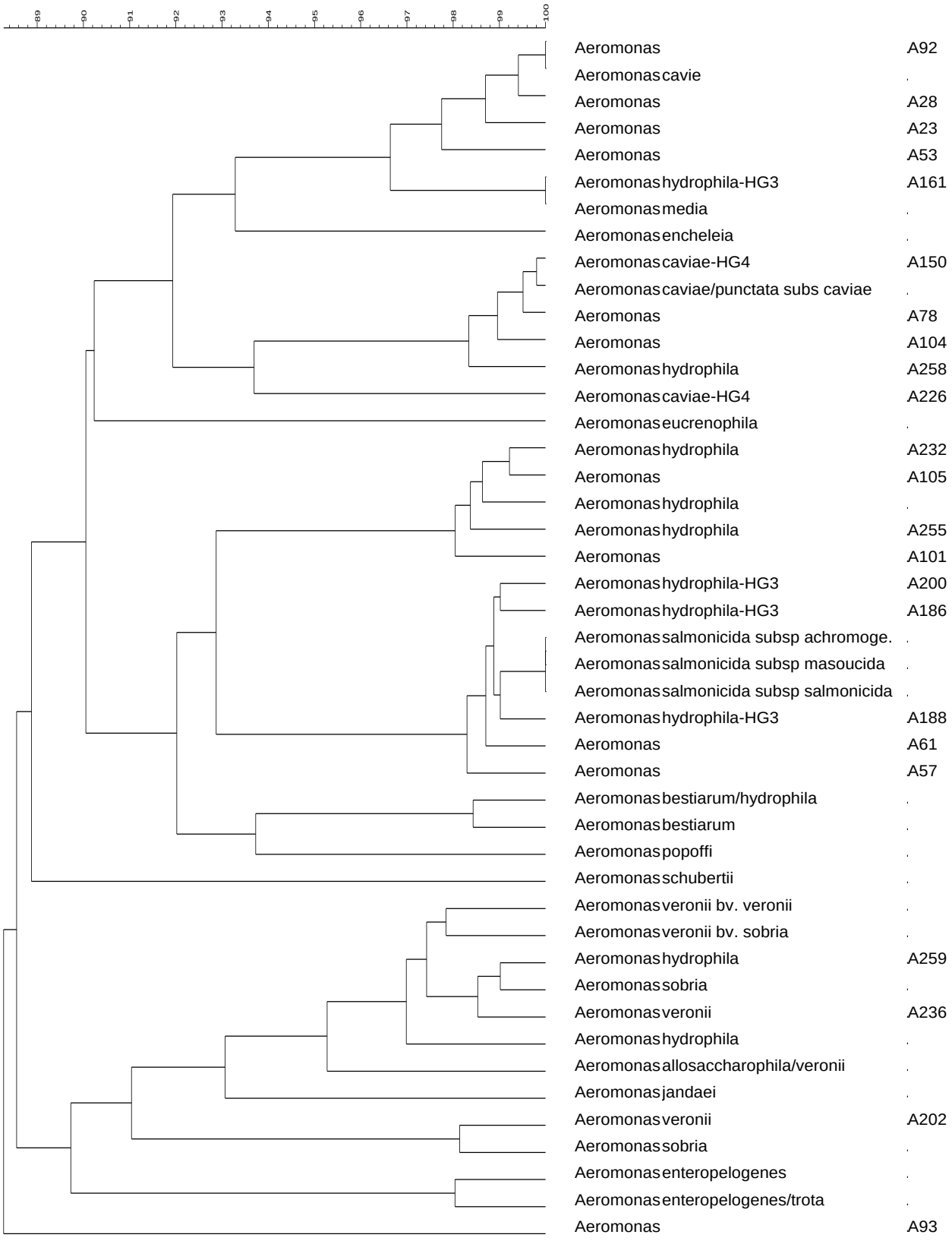

Figure 11 - Dendrogram obtained by multiple alignment of the concatenated sequences of *groL* gene and grouped by the agglomerative clustering of unweighted pair group method with arithmetic mean (UPGMA).

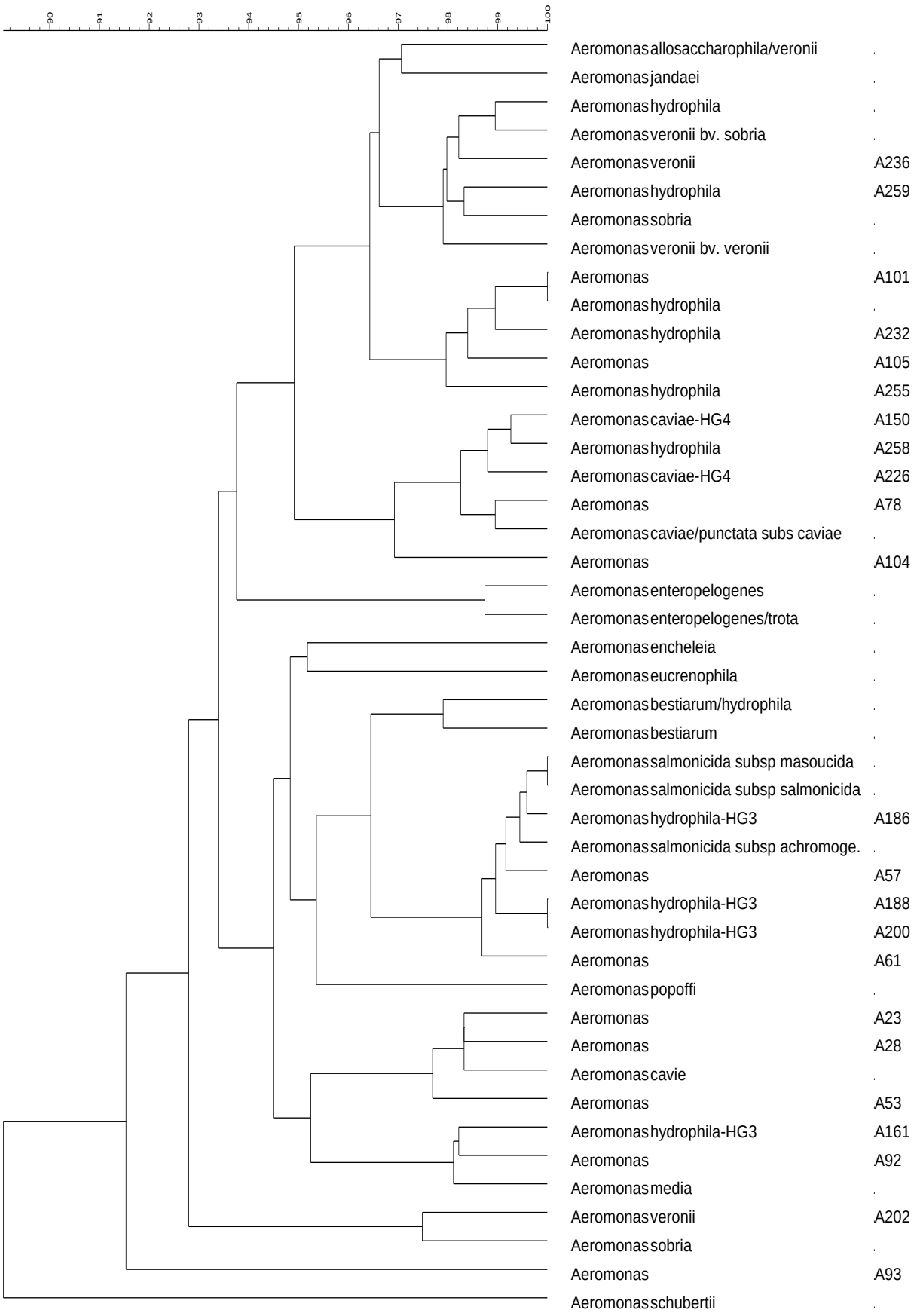

Figure 12 - Dendrogram obtained by multiple alignment of the concatenated sequences of *gyrB* gene and grouped by the agglomerative clustering of unweighted pair group method with arithmetic mean (UPGMA).

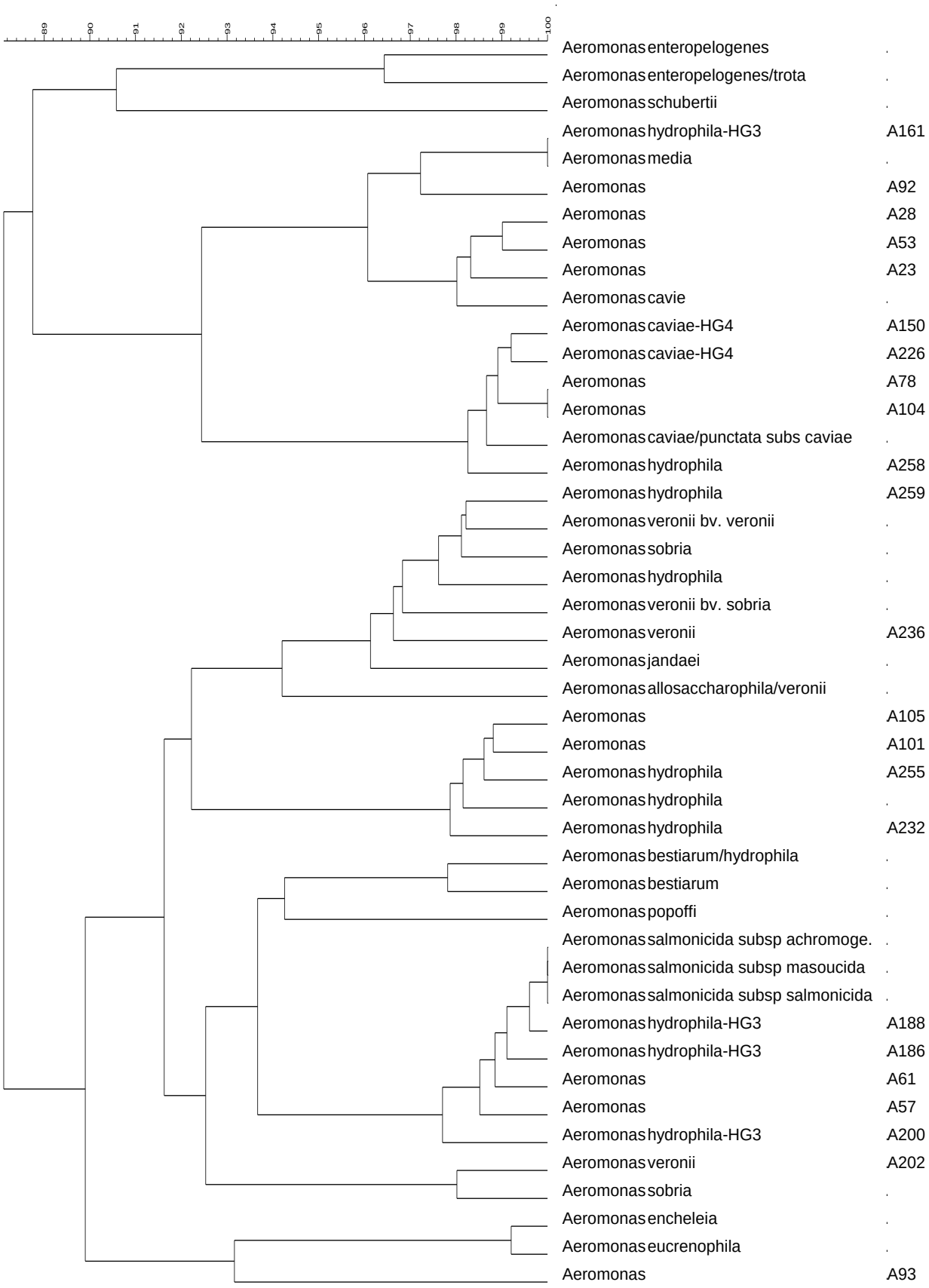

Figure 13 - Dendrogram obtained by multiple alignment of the concatenated sequences of *metG* gene and grouped by the agglomerative clustering of unweighted pair group method with arithmetic mean (UPGMA).

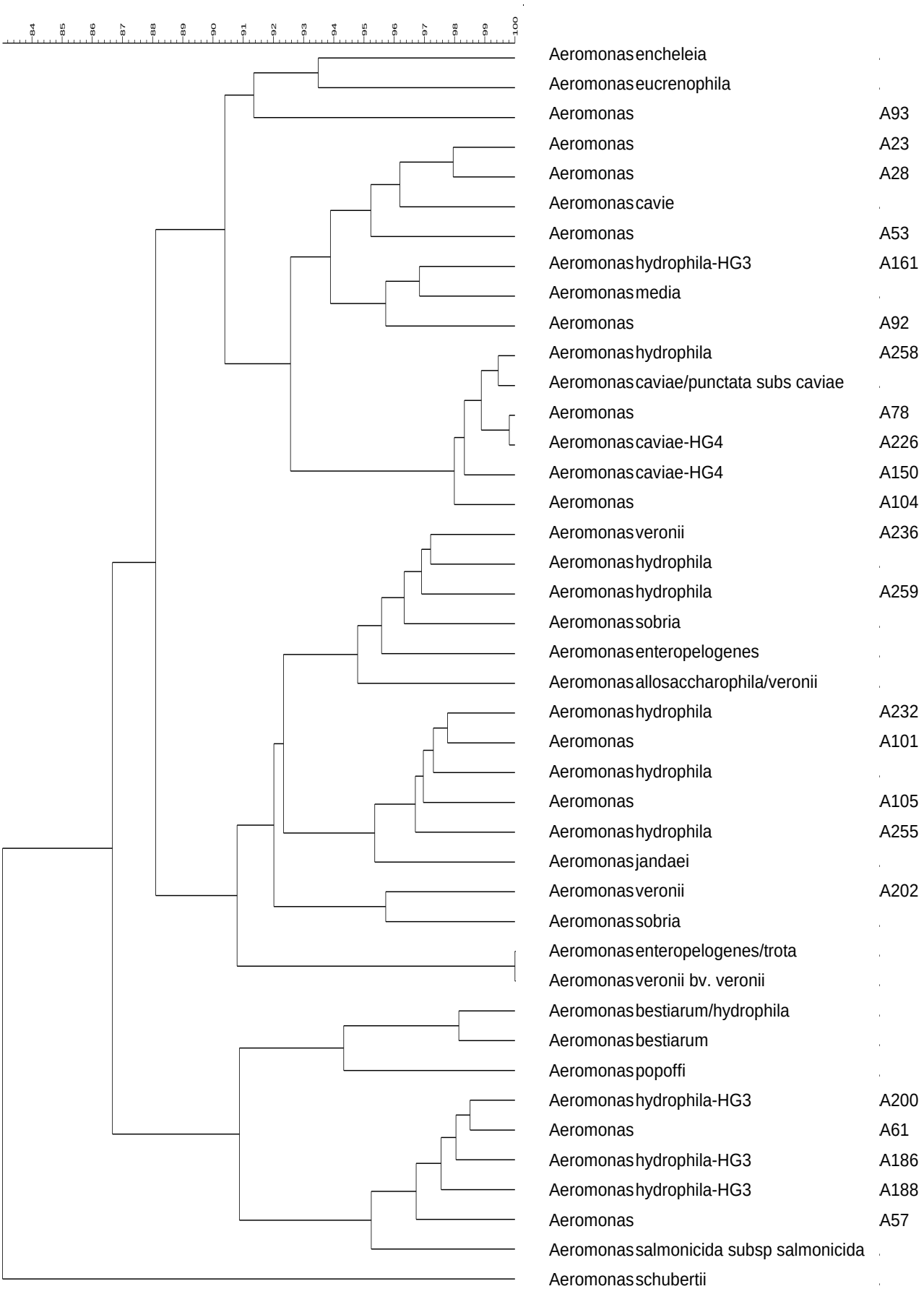

Figure 14 - Dendrogram obtained by multiple alignment of the concatenated sequences of *ppsA* gene and grouped by the agglomerative clustering of unweighted pair group method with arithmetic mean (UPGMA).

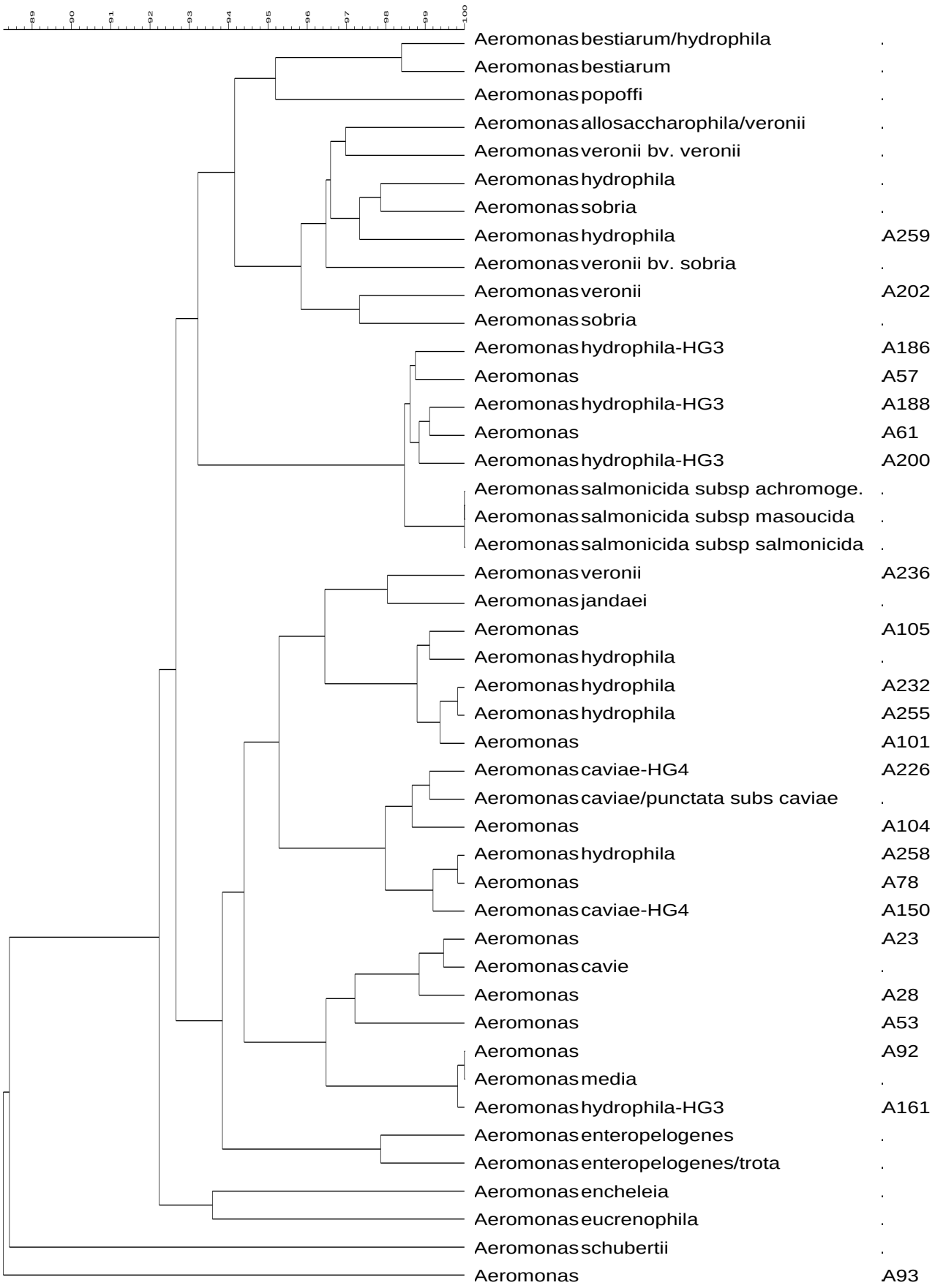

Figure 15 - Dendrogram obtained by multiple alignment of the concatenated sequences of *recA* gene and grouped by the agglomerative clustering of unweighted pair group method with arithmetic mean (UPGMA).